The epidemiology of trypanosomiasis, a re-emerging zoonosis in Uganda

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Submitted in fulfilment of the Requirements for the Degree of Doctor of Philosophy

The University of Edinburgh
2002
I declare that the research described within this thesis is my own work and that the thesis is my own composition.
“Questions of the animal origins of human disease lie behind the broadest pattern of human history, and behind some of the most important issues in human health today.”

Jared Diamond, in: Guns, germs and steel: a short history of everybody for the last 13,000 years (p197; Vintage Press).
# Table of Contents

Abstract.......................................................................................................................... 1
Résumé............................................................................................................................... 2
Acknowledgements ........................................................................................................... 3
List of Figures................................................................................................................... 5
List of Tables.................................................................................................................... 7
Abbreviations used ......................................................................................................... 8

Chapter I: General introduction ..................................................................................... 9

1.1 Zoonoses, emerging zoonoses and public health...................................................... 10
1.2 Trypanosomes: basic biology and distribution ......................................................... 12
   1.2.1 *T. rhodesiense* and *T. gambiense* ................................................................. 13
1.3 Description of southeast Uganda and the sleeping sickness foci............................ 15
   1.3.1 Administrative structure ................................................................................. 15
1.4 Hosts and reservoirs of *T. brucei* s.l. ................................................................... 17
   1.4.1 Wild hosts ....................................................................................................... 17
   1.4.2 Domestic hosts ............................................................................................... 18
1.5 Hosts of *Glossina* ................................................................................................. 20
1.6 Treatment of trypanosomiasis.................................................................................. 21
   1.6.1 Treatment of human trypanosomiasis ............................................................. 21
   1.6.2 Treatment of bovine trypanosomiasis ............................................................. 21
   1.6.3 Treatment of zoonotic trypanosome species ................................................. 22
1.7 Disease burden imposed by human sleeping sickness ........................................... 22
   1.7.1 GBD study ..................................................................................................... 22
   1.7.2 *T. rhodesiense* specific DALY in Uganda .................................................. 23
1.8 Importance of sleeping sickness research ............................................................... 24

Chapter II: Cattle movements and the spread of disease .............................................. 26

2.1 Introduction............................................................................................................... 27
   2.1.1 The Karimojong and raiding ......................................................................... 27
   2.1.2 Restocking ..................................................................................................... 28
   2.1.3 Hypothesis and specific aims ....................................................................... 29
   2.1.4 Risk assessments ......................................................................................... 30
      2.1.4.1 Stochastic or deterministic assessments ................................................. 30
      2.1.4.2 Case studies .......................................................................................... 31
2.2 Methods .................................................................................................................. 34
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1 Cattle and human population interactions</td>
<td>34</td>
</tr>
<tr>
<td>2.2.2 Cattle tracing</td>
<td>34</td>
</tr>
<tr>
<td>2.2.3 Risk assessment</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3.1 Data quality and parameter estimation</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3.2 Monthly imports</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.2.1 Kimwanga market records</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.2.2 Official imports</td>
<td>37</td>
</tr>
<tr>
<td>2.2.3.2.3 Unofficial imports</td>
<td>37</td>
</tr>
<tr>
<td>2.2.3.3 Proportion of <em>T. brucei</em> s.l. infections</td>
<td>37</td>
</tr>
<tr>
<td>2.2.3.4 Proportion of <em>T. b. rhodesiense</em> infections</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.5 Risk reduction</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.5.1 Effect of screening and selective treatment</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.5.2 Effect of mass treatment</td>
<td>39</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>43</td>
</tr>
<tr>
<td>2.3.1 Broad-scale relationships</td>
<td>43</td>
</tr>
<tr>
<td>2.3.2 Cattle tracing</td>
<td>44</td>
</tr>
<tr>
<td>2.3.3 Risk assessment: disease introduction</td>
<td>44</td>
</tr>
<tr>
<td>2.3.4 Risk assessment: disease control</td>
<td>47</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>49</td>
</tr>
<tr>
<td>2.4.1 Importation and transmission</td>
<td>49</td>
</tr>
<tr>
<td>2.4.2 Interventions</td>
<td>51</td>
</tr>
<tr>
<td>2.4.2.1 SRA</td>
<td>53</td>
</tr>
<tr>
<td>2.4.3 Cost-effectiveness of interventions</td>
<td>53</td>
</tr>
</tbody>
</table>

**Chapter III: A new disease focus: establishment and expansion** 56

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Introduction</td>
<td>57</td>
</tr>
<tr>
<td>3.1.1 Background</td>
<td>57</td>
</tr>
<tr>
<td>3.1.1.1 Soroti district</td>
<td>57</td>
</tr>
<tr>
<td>3.1.1.2 Sleeping sickness in Soroti</td>
<td>57</td>
</tr>
<tr>
<td>3.1.2 Hypothesis</td>
<td>59</td>
</tr>
<tr>
<td>3.1.3 Case control studies</td>
<td>59</td>
</tr>
<tr>
<td>3.1.4 Spatial statistics</td>
<td>60</td>
</tr>
<tr>
<td>3.1.5 The concept of spatial clustering</td>
<td>60</td>
</tr>
<tr>
<td>3.1.6 Moving window statistics</td>
<td>61</td>
</tr>
<tr>
<td>3.1.6.1 The Kulldorff and Nagarwalla spatial scan statistic</td>
<td>62</td>
</tr>
<tr>
<td>3.1.6.1.1 Bernoulli or Poisson</td>
<td>62</td>
</tr>
<tr>
<td>3.1.6.1.2 Examples: human medicine</td>
<td>63</td>
</tr>
<tr>
<td>3.1.6.1.3 Examples: animal health studies</td>
<td>64</td>
</tr>
<tr>
<td>3.1.7 Definitions: sleeping sickness in Soroti</td>
<td>64</td>
</tr>
<tr>
<td>3.2 Methods</td>
<td>66</td>
</tr>
<tr>
<td>3.2.1 Field</td>
<td>66</td>
</tr>
<tr>
<td>3.2.1.1 Cases</td>
<td>66</td>
</tr>
<tr>
<td>3.2.1.2 Controls</td>
<td>66</td>
</tr>
<tr>
<td>3.2.1.3 Georeferenced data</td>
<td>67</td>
</tr>
<tr>
<td>3.2.2 Statistical analyses</td>
<td>68</td>
</tr>
<tr>
<td>3.2.2.1 Distance varying with time</td>
<td>68</td>
</tr>
</tbody>
</table>
Chapter IV: A new disease focus: strain typing................................. 81

4.1 Introduction................................................................. 82
  4.1.1 The available toolbox............................................ 83
    4.1.1.1 Human serum resistance................................. 83
    4.1.1.2 Isoenzymes electrophoresis............................. 84
    4.1.1.3 Restriction Fragment Length Polymorphisms........... 85
      4.1.1.3.1 RFLP studies in Uganda.......................... 86
    4.1.1.4 PCR.......................................................... 87
    4.1.1.5 Mobile genetic elements................................. 88
      4.1.1.5.1 RIME.................................................. 89
      4.1.1.5.2 The MGE-PCR concept............................... 89
  4.1.2 Population structure............................................ 91
  4.1.3 Specific objectives.............................................. 93

4.2 Methods ............................................................... 95
  4.2.1 Sample collection................................................. 95
  4.2.2 Parasite amplification.......................................... 97
  4.2.3 Parasite extraction.............................................. 97
  4.2.4 Concentration of parasites.................................... 99
  4.2.5 DNA extraction.................................................. 99
  4.2.6 DNA extraction from procyclic stocks....................... 100
  4.2.7 MGE-PCR......................................................... 100
  4.2.8 Electrophoresis.................................................. 102
  4.2.9 Measurement of banding patterns............................ 102
  4.2.10 Phylogenetic analysis......................................... 102

4.3 Results................................................................. 104
  4.3.1 In vivo parasite amplification.................................. 104
  4.3.2 DNA concentration issues...................................... 105
  4.3.3 MGE-PCR gels.................................................... 105
  4.3.4 Banding patterns and phylogenetic analysis.................. 108
    4.3.4.1 T. b. rhodesiense stocks: Group 3...................... 108
      4.3.4.1.1 Busoga/Tororo..................................... 108
      4.3.4.1.2 Soroti.............................................. 108
    4.3.4.2 T. b. brucei stocks: Group 2............................. 109
      4.3.4.2.1 MULC22............................................. 109
    4.3.4.3 T. b. brucei stocks: Group 1............................. 110

4.4 Discussion............................................................ 115
Abstract

Sleeping sickness caused by Trypanosoma brucei rhodesiense and transmitted by tsetse flies (Glossina spp.) is a re-emerging zoonotic disease in eastern Africa. The disease may occur in large scale epidemics or be maintained at endemic levels, but in both cases, it is transmitted by the fly vector from the animal reservoir to humans. In Uganda, the principal animal reservoir is domestic cattle.

This thesis explores a number of components of T.b. rhodesiense epidemiology in Uganda. Firstly, unrest and cattle raids in some regions of Uganda has led to the depletion of the cattle population, which has subsequently been restocked, resulting in a mobile national cattle herd. Following an outbreak of sleeping sickness in the previously unaffected district of Soroti, eastern Uganda, retrospective data were collected from cattle markets and used to assess the risk, over the period of restocking, of importing cattle infected with T.b. rhodesiense. Secondly, a case-control study is presented, in which the epicentre of the Soroti outbreak and the temporal and spatial changes in the distribution of the affected villages are quantified. Thirdly, molecular epidemiological tools were used to characterise parasite isolates derived from the Soroti outbreak, and, by comparing their genetic structure to parasites collected in other regions, the parasite populations from which they were derived have been determined, and aspects of the population structure of trypanosomes more generally discussed. Finally, a retrospective analysis of clinical data from the 1900-1910 sleeping sickness epidemic in southern Uganda was carried out. There has been some controversy over the species of parasite involved in this outbreak (T. b. rhodesiense or Trypanosoma brucei gambiense), and the analysis of the historical records definitively addresses this question.

The risk analysis using the cattle market data shows that in Soroti, 54% of animals traded in the market in question originated from outside the district, from known sleeping sickness risk areas, and that up to 12.5% of the monthly imports from these areas may have been carriers of T.b. rhodesiense parasites. The theoretical impact that the alternative control options of mass chemotherapy or selective treatment following screening by microscopy would have had on reducing this risk are also considered, highlighting the limitations of field microscopy as a diagnostic tool for trypanosomiasis. Results from the case-control study showed that the epicentre of the outbreak which started in 1998 was Brookes Corner cattle market, the site through which most of the cattle in the restocking programme passed. Although residence in a village in proximity to the market was a highly significant risk factor for becoming a sleeping sickness case at the start of the outbreak, the average distance of cases to the market increased with time, indicating that the outbreak is expanding away from that point source. The results of the molecular analyses confirm that the parasites circulating in Soroti were similar to strains from the established sleeping sickness regions further south in Uganda, and shed further light on the nature of trypanosome population structures. T.b. rhodesiense and T.b. gambiense are distinguished primarily by the differences in clinical presentation; comparing the 1900-1910 sleeping sickness data to a contemporary dataset of T.b. rhodesiense cases shows clearly that the clinical course of the disease in 1900-1910 was identical to that seen at present in Uganda. These results confirm that T.b. rhodesiense was present in Uganda at the time.

The results are discussed in relation to the management of human trypanosomiasis in the cattle reservoir, with particular attention given to the implications of trypanosomiasis control polices aimed at livestock but which also benefit the health of the human population. Policies aimed at controlling disease in this way need to be made at a national level, and the importance of collaboration between medical and veterinary authorities for zoonotic disease control is stressed.
Résumé

La maladie du sommeil, provoquée par *Trypanosoma brucei rhodesiense* et transmise par la mouche tse-tse (*Glossina* spp.) est une zoonose ré-émergente en Afrique de l’est. La maladie peut survenir en grandes épidémies ou se maintenir à l’état endémique, mais dans les deux cas, elle est transmise par la mouche depuis le réservoir animal vers les êtres humains. En Ouganda, le principal réservoir animal est constitué par le cheptel domestique.

Cette thèse examine différents aspects de l’épidémiologie de la maladie. En premier lieu, il est décrit comment des troubles sociaux et des vols de bétail dans certaines régions de l’Ouganda ont provoqué l’épuisement des populations de bétail qui ont été ensuite reconstituées, la résultante étant un troupeau mobile de bétail à l’échelon national. A la suite d’un début d’épidémie de maladie du sommeil dans le district de Soroti en Ouganda oriental, qui jusque là n’en avait jamais été affecté, des données rétrospectives ont été collectées sur les marchés de bétail et utilisées pour évaluer le risque, pendant la période de restockage, de l’importation de bétail atteint de trypanosomiase. En deuxième lieu, l’auteur présente une étude quantitative de cas-témoins, à partir de l’épicentre de Soroti de la distribution des villages affectés, dans le temps et dans l’espace. En troisième lieu, état est fait des outils d’épidémiologie moléculaire, utilisés pour caractériser les souches de parasites dérivées de l’épidémie de Soroti, et en comparant leur structure génétique avec celles de parasites collectés dans d’autres régions, la population de parasites dont elles étaient dérivées a été déterminée. Finalement, une analyse rétrospective des cas relevés pendant l’épidémie de maladie du sommeil en Ouganda au sud pendant la période 1900 à 1910 a été effectué. Une controverse existe quand à l’espèce de parasite concerné par cette épidémie (*T.b. rhodesiense* ou *Trypanosoma brucei gambiense*), et l’analyse historique des archives adresse cette question de façon définitive.

L’analyse du risque utilisant les données des marchés de bétail montre qu’à Soroti, 54% du bétail échangé sur le marché en question provenait de l’extérieur du district, et de régions connues comme étant des zones à risque pour la maladie du sommeil, et que jusqu’à 12,5% des importations mensuelles depuis ces régions pouvaient avoir testé positif pour le parasite *T.b. rhodesiense*. L’impact théorique pour la réduction du risque entre les options de contrôle par chimiothérapie massive et par traitement sélectif faisant suite à un examen par microscopie, a aussi été comparé, tout en faisant ressortir les limites de l’emploi de la microscopie sur le terrain en tant qu’outil de diagnostique pour la trypanosomiase. Les résultats de l’étude de cas-témoins ont montré que l’épicentre de l’épidémie de 1998 était le marché de bétail de Brookes Corner, carrefour où avait transité la plupart du bétail pendant le programme de repopulation. Alors que le fait de résider dans un village à proximité de ce marché représentait un risque significatif d’être victime de l’épidémie à ses débuts, la distance entre les nouveaux cas et le marché allait en augmentant avec le temps, indiquant ainsi que l’épidémie s’étendait de son point d’origine. Les résultats de l’analyse moléculaire confirment que les parasites qui circulaient à Soroti étaient similaires aux types provenant de régions où la maladie du sommeil était établie plus au sud de l’Ouganda, et éclairent la nature des structures de la population de trypanosome de façon plus générale. *T.b rhodesiense* et *T.b gambiense* sont distingués essentiellement par des différences de présentation clinique. Une comparaison des données de l’épidémie de maladie du sommeil de 1900-1910 avec les données contemporaines de cas dus au *T.b. rhodesiense* montre clairement que le cours clinique de la maladie de 1900-1910 était identique au cours de la maladie actuellement observée en Ouganda. Ces résultats confirment que *T.b rhodesiense* était présent en Ouganda à cette époque.

Les résultats sont discutés en relation avec le traitement de la trypanosomiase dans le réservoir de bétail, et une attention particulière est portée à l’implication des politiques de contrôle du bétail qui pourront aussi bénéficier à la santé des populations humaines. Les politiques destinées à un tel contrôle de la maladie doivent être élaborées à l’échelon national et l’importance de la collaboration entre les autorités vétérinaires et les autorités médicales dans le contrôle des zoonoses est soulignée.
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List of Figures

Figure 1.1: Officially recorded cases of sleeping sickness in Africa ........................................ 11
Figure 1.2: Recognised foci of Gambian (west) and Rhodesian (east) sleeping sickness .................................................. 12
Figure 1.3: The transmission cycle of T.b. rhodesiense ................................................................. 14
Figure 1.4: District boundaries in Uganda ...................................................................................... 16
Figure 2.1: Event tree showing risk events modelled in @Risk ......................................................... 40
Figure 2.2: The hypothetical effects of two potential interventions ..................................................... 41
Figure 2.3: Origins of cattle traded at Brookes Corner during the study period ............................. 43
Figure 2.4: a) Monthly and b) cumulative imports of T.b. rhodesiense-positive animals to Brookes Corner market ........................................... 46
Figure 2.5: Proportion of all imports that are T.b. rhodesiense positive. ............................................ 47
Figure 2.6: Cumulative number of T.b. rhodesiense-positive animals entering Brookes Corner ......................................................... 48
Figure 3.1: Sleeping sickness cases in Soroti, December 1998 – August 2001. ............................. 70
Figure 3.2: Age distribution of sleeping sickness cases in Serere ...................................................... 71
Figure 3.3: Results of the ANCOVA analysis ....................................................................................... 72
Figure 3.4: Cluster centre during the first month (January 1999) of the outbreak ........................ 75
Figure 3.5: Cluster centres and cluster area at different time points .................................................. 76
Figure 3.6: Map of Uganda showing the distribution of G. f. fuscipes ............................................. 79
Figure 4.1: Diagram of the amplification of mobile elements using single primers for the MGE-PCR ........................................................................................................... 90
Figure 4.2: Area of eastern Uganda from which samples were collected ........................................ 95
Figure 4.3: A typical MGE-PCR gel using the REV B primer .......................................................... 106
Figure 4.4: MGE-PCR gel using the REV B primer ........................................................................... 107
Figure 4.5: Dendrogram resulting from the UPGMA cluster analysis ........................................... 114
Figure 4.6: Sleeping sickness positive villages in Tororo District. ..................... 117

Figure 5.1: Age distribution of 204 sleeping sickness cases in Mengo .................. 133

Figure 5.2: Outcome of admission for 204 probable sleeping sickness patients reporting to the Mengo hospital between 1901-1910. ........................................... 134

Figure 5.3: Kaplan-Meier survival curves ............................................................... 136
List of Tables

Table 1.1: Disability weights and type of disability associated with each weight ... 23
Table 2.1: Relevant definitions relating to the study of risk.......................... 31
Table 2.2: Properties of each step in the @Risk model.................................. 42
Table 3.1: Projection details for the National Biomass Study dataset ............... 67
Table 3.2: Parameter estimates from the conditional logistic regression .......... 73
Table 3.3: Spatial scan statistic results .......................................................... 74
Table 4.1: Isoenzymes used by Hide et al. (1994)........................................ 85
Table 4.2: Recipes for solutions used in the preparation and storage of trypanosome material ................................................................. 98
Table 4.3: History of purified samples collected between 1998 and 2000............. 104
Table 4.4: Presence or absence of bands of each size for each sample, using the REV B primer ................................................................. 111
Table 5.1: Comparative data of duration of illness to death of sleeping sickness patients in Tororo, 1988-1990......................................................... 130
Table 5.2: T.b. gambiense cases with reported period of infection..................... 131
Table 5.3: Time sick pre-admission and total time to death for 12 patients admitted between 1901 and 1910 ......................................................... 134
Abbreviations used

BCT    Buffy Coat Technique
BIIT   Blood Incubation Infectivity Test
BSE    Bovine Spongiform Encephalopathy
CMS    Church Missionary Society
CNS    Central Nervous System
CTVM   Centre for Tropical Veterinary Medicine
DALY   Disability-Adjusted Life Year
DEAE   Diethylaminoethyl
DNA    Deoxyribose Nucleic Acid
COCTU  Co-ordinating Office for the Control of Trypanosomiasis in Uganda
GIS    Geographical Information System(s)
GPS    Global Positioning System
HCT    Haematocrit Centrifugation Technique
HSR    Human Serum Resistance
IFAT   Indirect Fluorescent Antibody Technique
ILRI   International Livestock Research Institute
LIRI   Livestock Research Institute
MGE    Mobile Genetic Element
PBS    Phosphate-Buffered Saline
PBSGH  PBS with Glucose and Heparin
PSG    Phosphate-buffered Saline with Glucose
PCR    Polymerase Chain Reaction
RFLP   Restriction Fragment Length Polymorphism
RIME   Ribosomal Mobile Element
RNA    Ribonucleic Acid
SRA    Serum Resistance-Associated protein
UPGMA  Unweighted Pair Group Method using Arithmetic averages
UV     Ultra-Violet
vCJD   new-variant Creutzfeldt-Jakob Disease
WHO    World Health Organization
Chapter I: General introduction
1.1 Zoonoses, emerging zoonoses and public health

Zoonoses are defined as "diseases and their agents which are naturally transmitted between (other) vertebrate animals and man" (WHO, 1959), and can be either so called accidental infections, or those for which humans are an obligate part of the life cycle. Emerging and re-emerging infectious diseases are defined as communicable infections of humans which are either newly recorded as infections, or more generally for which the incidence has increased exceptionally in the past two decades (CDC, 1994). A recent study (Taylor et al., 2001) was the first to comprehensively enumerate all human infectious diseases, and concluded that of 1415 organisms identified as causative of disease in humans, 61% were zoonotic; zoonotic species are, in addition, twice as likely to be associated with emerging or re-emerging diseases than non-zoonotic species. Public health efforts therefore need to be directed at this important group of pathogens. Recently, interest in such diseases has grown at the international level, with institutions such as the World Health Organization (WHO) and the U.S. Centres for Disease Control (CDC) making surveillance, applied research and control activities targeted at them a priority (CDC, 1994; Meslin, 1997).

The factors underlying disease (re)emergence are probably only partly related to virulence of the organisms or other biological factors directly related to the pathogens themselves. Significant social issues (e.g. civil unrest and poverty, international travel), the breakdown of control programmes (often due to lack of funds), resistance to anti-microbial treatments and resistance to pest control compounds such as insecticides, ecological changes (often anthropogenic environmental change) or other emerging diseases (e.g. HIV/AIDS), may allow pathogens to emerge or re-emerge (Morse, 1995; Schrag & Wiener, 1995; Heymann, 1997). An important factor leading to disease (re)emergence, for zoonoses or infectious diseases in general, is the contact of human populations with habitats marginal to the distribution of humans. There are many examples of this, such as the emergence of lyme disease in the USA (Dister et al., 1997; Kitron & Kazmierczak, 1997; Kitron, 1998), Echinococcus multilocularis in central China (Craig et al.,
malaria in the forest regions of southeast Asia (Fèvre et al., 1999; Kidson et al., 1999), leishmaniasis in many regions (Ashford, 2000) and finally Kyasanur Forest Disease virus in India (LeDuc, 1989). Understanding the extent to which these diseases affect rural communities and the significance of the zoonotic reservoir in the maintenance and propagation of the causative organisms, is fundamental to the design and implementation of control programmes.

Trypanosomiasis is a further example of a re-emerging disease (Molyneux, 2001b). The incidence of infection with the causative organism has been increasing during the second half of the twentieth century (see Figure 1.1). In Uganda, for example, the parasite has recently spread to previously unaffected areas in the east of the country. The investigation of that spread makes up the bulk of this thesis. In other regions, such as Tanzania’s Serengeti, sleeping sickness was recorded from the 1920s onwards (Geigy et al., 1971; Onyango et al., 1971), and following a long period of apparent absence, cases have occurred recently, highlighted by a number of infections of tourists visiting the area (Jones, 2000; Moore et al., 2002).

![Figure 1.1: Officially recorded cases of sleeping sickness in Africa from 1941 to 1998, showing the extent of disease re-emergence in recent years; the reduction in number of people screened is indicative of high under-reporting rates. Screening in this context is by active case detection in affected areas. Data reproduced with permission from WHO.](image-url)
1.2 Trypanosomes: basic biology and distribution

Trypanosomes are kinetoplastid protozoan parasites, with a wide potential host range (see Section 1.4). In humans, two species-groups are of importance, *Trypanosoma cruzi*, transmitted by reduviid bugs (*Triatoma* spp.) in South America, and *Trypanosoma brucei* s.l., which is restricted to sub-Saharan Africa. There are two sub-species of African human trypanosomes, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. Generally, *T.b. gambiense* occurs in west and central Africa and *T.b. rhodesiense* in East Africa (see Figure 1.2). A third sub-species of *T. brucei* s.l. is *Trypanosoma brucei brucei*, which is pan-African in distribution and which affects cattle, but is not pathogenic to East African breeds (Killick-Kendrick, 1971). Interestingly, the continental division between *T.b. gambiense* and *T.b. rhodesiense* follows very closely the line of the African Rift Valley. This and other biological differences between the parasites has led to the suggestion that trypanosomes may have played a role in the exodus of *Homo sapiens* from Africa (Welburn *et al.*, 2001a).

![Figure 1.2: Recognised foci of African sleeping sickness superimposed on African topography. The line divides the regions affected by Gambian (west) and Rhodesian (east) sleeping sickness, shown in yellow and red respectively. Foci data from WHO; USGS digital elevation data provided by Eric Augenstein of Earthstar Geographies (USA).](image-url)
1.2.1 *T. b. rhodesiense* and *T. b. gambiense*

*T. b. rhodesiense* is an acute infection in humans, the condition of the patient deteriorating rapidly as the parasite moves from the blood stream (early stage infection) through to the central nervous system (CNS), which is diagnostic of late stage infection. The pathology resulting from infection with the parasite is described in detail by Kristensson and Bentivoglio (1999). Briefly, initial infection results in a chance at the site of inoculation by the fly. The chancre, well illustrated by Moore et al. (2002), is a localised immune response resulting in inflammation. Parasites then move to the lymph nodes and are disseminated in the circulatory system, multiplying in both the blood and lymph. Following invasion of the CNS, inflammation of the brain tissue occurs (meningo-encephalitis), as well as cellular infiltration of spinal nerve tissue and inflammation of visceral organs. In the absence of treatment, death usually occurs within 6-8 months (Apted, 1970; Odiit et al., 1997a).

*T. b. gambiense*, by contrast, is usually described as a chronic infection, often with a long asymptomatic stage of several years, and a chronic meningoencephalitic condition during the late stage (Apted, 1970). The important epidemiological distinction between these clinical courses is the length of the incubation period and the degree of disability resulting from infection (see Section 1.7.1). Debilitating symptoms appear rapidly with *T. b. rhodesiense*, and human-tsetse-human transmission which would require the patient to venture into tsetse habitat, is unlikely to occur and thus to play an important epidemiological role. With *T. b. gambiense*, however, the long, asymptomatic incubation period and apparently restricted host range makes humans the most significant reservoir of parasites (Welburn et al., 2001a). As humans are the main reservoir of *T. b. gambiense*, the most successful disease control paradigm is human case finding and treatment. The detailed epidemiology of *T. b. gambiense*, which will not be considered further here, has been reviewed recently by Pépin and Médá (2001). Each year, over 300,000 cases of African sleeping sickness (due to both parasite species) are reported on the continent, although this is generally thought to be an under-estimate; 50 million people live in risk areas (WHO, 1986), and as many of these have poor access to health care facilities, under-reporting rates are likely to be high.
*T.b. gambiense* was first described by Forde (1902) and named *T. gambiense* by Dutton (1902). *T.b. rhodesiense*, then called *T. rhodesiense*, was first identified by Stephens and Fantham (1910), in Zambia, and was first incriminated explicitly as the cause of human sleeping sickness in Uganda by MacKichan (1944), in an outbreak in the Busoga region. The organism responsible for outbreaks prior to this in Uganda is a cause for discussion, and the issue is addressed in Chapter V. Sub-specific status for the human infective trypanosomes was proposed by Hoare (1966). The life cycle and morphology of the human infective trypanosomes, including the obligate fly stages, are well understood (Vickerman, 1985); the details of the developmental stages of the parasite are less important in the context of this thesis than the transmission cycle, and the transmission cycle of *T.b. rhodesiense* (Baker, 1974) is shown in Figure 1.3. The vectors of human and animal trypanosomiasis on the African continent are flies of the genus *Glossina*. In eastern Uganda, the fly species responsible is *Glossina fuscipes fuscipes*. Trypanosomes take approximately 20-40 days to develop in the tsetse host (Dale *et al.*, 1995) depending on the susceptibility of the fly, which remain infectious until the end of their lives, which last approximately 5-6 weeks in the field (Welburn & Maudlin, 1999).

![Figure 1.3: The transmission cycle of *T.b. rhodesiense*, after Baker (1974). In the context of this thesis, the detailed life cycle of trypanosomes with the various parasite stages, is not as significant as the transmission cycle. The former is well described in Vickerman (1985). *T.b. rhodesiense* and *T. b. brucei* occur in a tsetse-bovid-tsetse cycle. The occasional infection of humans with *T.b. rhodesiense* results in sleeping sickness. The nature of human-fly contact and the acute nature of the disease make it unlikely that humans act as a reservoir of infection. By contrast, in *T.b. gambiense*, which is chronic, and for which the role of an animal reservoir is uncertain, humans are the main reservoir (Welburn *et al.*, 2001a). See text for discussion.](image-url)
1.3 Description of southeast Uganda and the sleeping sickness foci

Uganda lies astride the Equator (1.5°S to 4.2°N, 29.3°E to 34.9°E). The south-eastern region is characterised by a bimodal rainfall cycle, with dry seasons between December and February and July to August. Peak rainfall is in the period April to May and September to October. Most of the populated area of eastern Uganda is at approximately 1100 metres above sea level, with drainage (other than the Nile River) mainly as sluggish streams and swamp networks (Abaru, 1985). Annual average rainfall in the region is approximately 1300 mm. and the maximum average temperature is approximately 30°C (Philips, 1997).

1.3.1 Administrative structure

The largest administrative unit in Uganda is the district, of which there are currently 45. Increasing moves towards decentralization as a consequence of public sector reform in the country is resulting in the creation of more districts by the subdivision of existing ones. Below the district, are the county, sub-county, parish and village levels. Each level is represented by a chairman, LC1 for the village up to LC5 for the district. The health infrastructure is presently based on health sub-districts (Jeppsson, 2001), which, rather than corresponding to existing administrative boundaries, correspond to the distribution of the population, such that health service provision is evenly distributed.

Trypanosomiasis caused by *T. congoense*, *T. brucei* s.l. and *T. vivax* all occur in the domestic cattle population in south-east Uganda (P.G. Coleman, unpublished; E.M. Fèvre, pers. obs.). In terms of human trypanosomiasis, Uganda has the misfortune of suffering from both *T.b. gambiense* and *T.b. rhodesiense*, although within Uganda, the former occurs only in the north west of the country, west of the river Nile. *T.b. rhodesiense* occurs in the south-east (see Figure 1.4). Sleeping sickness has been recorded in Uganda since the start of the last century; the pattern of disease occurrence has been defined by large epidemics separated by long endemic periods. The most recent epidemic started in Kamuli, Iganga, Bugiri and Jinja districts – the
Busoga region – in the last years of the 1970s. In the 14 years up to 1990, 40,000 cases were confirmed parasitologically in Busoga, with 6,000 additional cases in surrounding regions (Smith et al., 1998).

Figure 1.4: District boundaries in Uganda, highlighting (in green) those districts considered to be part of the “established foci” of sleeping sickness. Blue shaded areas are areas of open water and extensive swamp. The red dot is the location of Serere Health Centre (see Chapter III).
The first cases of sleeping sickness seen in Tororo district (now separated into Tororo and Busia districts) were in the mid 1980s. Since 1987, over 12,000 cases have occurred in these two districts alone. The mechanism by which sleeping sickness came into Tororo and Busia districts has not been investigated, but it is likely that it was the result of an expansion eastwards of the Busoga focus to the West. An outbreak in Soroti, discussed in Chapters II-IV, presents an opportunity to study the spread of the disease in a way that was not possible previously, for reasons linked to politics, advances in geographical data handling and limitations in the technology available for the analysis of parasite strains.

Sleeping sickness incidence in Uganda varies annually. In 1998, during the current "endemic period," a total of 1,247 cases were reported nationwide (COCTU, 1998). Of these, 979 patients (78.5%) suffered from T.b. gambiense in the northwest (including Sudanese refugees), and 268 (21.5%) suffered from T.b. rhodesiense in the southeast of the country (including 15 Kenyan nationals who reported to Ugandan health services and were treated by them).

1.4 Hosts and reservoirs of T. brucei s.l.

1.4.1 Wild hosts

Traditionally, the bushbuck (Tragelaphus scriptus) has been considered the archetypal animal reservoir host for T.b. rhodesiense, as a result of the fact that a human infective trypanosome was first isolated from this species by Heisch and others (1958). Other wild animals, especially bovids, may also be hosts (Geigy et al., 1971). These may include hartebeest (Alcelaphus buselaphus) and lion (Panthera leo). In Uganda, there has been a drastic change in land use as pressure to grow more food and more cash crops has increased since the 1940s. This, in combination with political factors which influence land tenure and occupancy, has resulted in changes in the natural environment. Although MacKichan (1944) observed that game was plentiful in the mid 1940s, southeast Uganda currently supports very little wild game and correspondingly few wild species of bovids (Eltringham & Malpas, 1993). Few formal studies have been undertaken on this
subject, but the work by Eltringham and Malpas (op. cit) relating to encroachment in game reserves shows a reduction in natural forest cover and wild species abundance, and can be taken as indicative of the general situation in agricultural areas of Uganda. Work in south-western Ethiopia also suggests that anthropogenic changes are having an effect on the tsetse population and consequently on sleeping sickness. Some *Glossina* species that occurred there are no longer present, and game animals are being hunted out to make room for raising domestic stock (Nigatu *et al.*, 1992). This part of Ethiopia may be a model for the changes that have already taken place in Uganda.

### 1.4.2 Domestic hosts

A number of domestic animal species, including pigs, dogs, goats, sheep and cattle may carry human infective trypanosome species (Stephen, 1970). Bruce and co-workers (1910; 1911) were the first to suggest that domestic animals might be important in the epidemiology of trypanosomiasis, and attached particular importance to cattle as a potential reservoir. Parasites isolated from domestic bovids were able to produce patent infections in monkeys (Bruce *et al.*, 1910). They state, fore-shadowing the development of the veterinary public health discipline, that

> "no branch of sleeping sickness investigation is of greater practical importance than the identification of animals which can serve as reservoirs of the sleeping sickness parasite" (p.236).

Onyango *et al.* (1966) isolated *T. brucei* s.l. parasites from domestic cattle and successfully infected a human, showing for the first time, that *T. brucei* s.l. had zoonotic potential (that is, their isolate was *T.b. rhodesiense*). Their study was conducted on the Kenyan shores of Lake Victoria, where a prevalence of *T. brucei* in cattle of 21.2% was found (Onyango *et al.*, 1966). Large numbers of cattle are kept in East Africa, in intimate contact with humans. Both humans and cattle may also have close contact with tsetse fly habitat. This makes cattle a potentially important reservoir of parasites. Maudlin *et al.* (1990) found that 4.9% of cattle tested in Ilyowa county, southeast Uganda during 1988 were carrying *T. brucei* s.l. infections,
and 25% of these were human serum resistant, indicating that they were probably *T.b. rhodesiense*. In contrast to the above, Okia *et al.* (1994) insist that cattle do not play an important role in the epidemiology of human sleeping sickness. They questioned sleeping sickness patients and controls in southeast Uganda on their behaviour, and observed that cases of sleeping sickness had fewer animals grazing near areas of human-tsetse contact than controls. Other species of trypanosomes of importance in Uganda, namely *T. vivax* and *T. congolense*, affect domestic cattle, pigs, sheep and goats (Stephen, 1986).

If cattle are indeed a significant reservoir of human infective trypanosomes, treating them, in the context of a bovine trypanosomiasis control programme, could theoretically prevent human disease. Welburn *et al.* (2001a) have modelled the impacts of this control technique, and found that it would have a significant public health impact, in an established sleeping sickness focus. The model was based on a previous model of trypanosome transmission (Rogers, 1988). It is a one vector (tsetse) and two host model (humans and cattle); Welburn *et al.* (*op. cit.*) add an extra component, that of the effects of drug treatment on both mammalian components of the life cycle. They concluded that if ≥ 60% of infected cattle are treated soon after becoming infected with *T. brucei*, and if human treatments are maintained at a high enough level (20% of cases) to prevent them from becoming a reservoir, the outbreak will die out as the number of flies picking up infections and infecting both humans and cattle with the parasites, will not be high enough. An important concept in the study of infectious disease epidemiology is $R_0$, the basic reproduction number (Anderson & May, 1992). If $R_0$ is less than 1 ($R_0<1$), then each infected individual gives rise, on average, to less than one new infection, and an outbreak would not sustain itself. For values of $R_0$ greater than 1 ($R_0>1$), the opposite is true as each infected individual gives rise to more than one new infection. Reducing the size of the animal reservoir in order to minimise the chances of human hosts becoming infected with trypanosomes, as described by Welburn *et al.* (2001a), is the application of the concept - keeping the number of infective vectors low enough such that transmission of the disease is interrupted.

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1 The article cited was partly the result of work carried out during the course of PhD study.
1.5 Hosts of Glossina

Blood meal analysis is the most objective method for identifying natural sources of food for tsetse (Weitz, 1970), and Weitz (op. cit.) notes that *G. f. fuscipes*, which is a riverine fly, has adapted to feed on a variety of hosts due to the nature of the ecological niche it occupies. Okoth and Kapaata (1988) studied the hosts of *G. f. fuscipes* during the recent epidemic in Busoga and Tororo by identifying the source of blood meals in wild caught flies. Humans, bushpig (*Potamochoerus porcus*), bushbuck and monitor lizards (*Varanus niloticus*) and bovids, including cattle, were the main sources of blood meals. Mohamed-Ahmed and Odulaja (1997), working in the Hoima Bay area of the Lake Victoria shore (in Kenya), reported a high prevalence of blood meals on monitor lizards (*Varanus spp.*) by *G. f. fuscipes* – up to 98.4%. The importance of the reptiles was emphasised by the concurrent low monitor lizard density in the study area. Gouteux et al. (1995) found that, compared to non-baited control traps, biconical traps baited with live monitor lizards caught 1.7 times more *G. f. fuscipes*, suggesting that the flies are in fact positively attracted to the reptiles rather than simply feeding on them opportunistically. Flies were also strongly attracted to cow urine, indicating a strong preference for these domestic animals. Jordan (1995) has reviewed the use of a range of attractants in tsetse control. Spath (2000), by contrast, working in West Africa, concludes that the principle motivation for a fly to feed on any particular host is the general availability of that host, and in an area where there are few mammals but abundant monitor lizards, these are likely to play a more significant role. Flies, in short, feed on what is available in their habitats. It should be noted that different species of fly in different habitats may have different behaviours, including feeding preferences.

Although the existence of a reptilian host for the maintenance of certain tsetse populations is likely, a reptilian contribution to the maintenance of trypanosomes is uncertain, but would raise important epidemiological questions. Under experimental conditions, *V. niloticus* maintained a patent infection of *T. brucei* for 2 weeks (Njagu et al., 1999). The animals were, however, kept at ‘ambient’ temperature (the actual temperature is not stated by the authors), which may not be representative of the field
situation in which wide temperature fluctuations might occur. In the same study, out of 19 lizards caught in Busia, Uganda, over 1.5 years, one was T. brucei positive. If temperatures are consistently within the range allowing T. brucei to survive, it is conceivable that the lizard could be a host. Further studies are required to quantify the potential contribution of reptiles to trypanosomiasis epidemiology.

1.6 Treatment of trypanosomiasis

1.6.1 Treatment of human trypanosomiasis

There are relatively few drugs available for treating sleeping sickness, and with all of these, there are issues of high toxicity, and hospitalisation is required during administration (WHO, 1995). The choice of treatment is dependent on the parasite species involved and on the stage of the disease; drugs for the treatment of late stage disease are designed to cross the blood brain barrier in order to clear trypanosomes in the cerebro-spinal fluid. T.b. rhodesiense is preferably treated with suramin or melarsoprol (an arsenical compound) in the early and late stages respectively, whereas T.b. gambiense is treated with suramin or pentamidine in the early stage and melarsoprol in late stage disease (WHO, 1995; Van Nieuwenhove, 1999). There are reports of clinical failures with melarsoprol in the treatment of T.b. gambiense (Burri & Keiser, 2001; Matovu et al., 2001a), but no significant reduction in efficacy has yet been demonstrated for T.b. rhodesiense. The alternative drugs for melarsoprol refractory-late stage T.b. gambiense treatment are nifurtimox or eflornithine (Van Nieuwenhove, 1999), but these cannot be used for T.b. rhodesiense.

1.6.2 Treatment of bovine trypanosomiasis

The drugs available for bovine use currently include the salts of only three compounds, videlicet diminazene aceturate, homidium bromide/homidium chloride and isometamidium chloride. Diminazine is used for curative rather than prophylactic treatment, while latter two drugs have a prophylactic effect. Trypanosomiasis control in cattle might be undermined by the development of resistance to these veterinary drugs (Geerts et al., 2001). No serious reports of drug
resistance have been made in Uganda; however, *T. congolense* resistance to isometamidium chloride has been reported in Kenya (Gray *et al.*, 1993) and to all available trypanocides in different parts of Ethiopia (Codjia *et al.*, 1993; Afewerk *et al.*, 2000), and *T. vivax* resistance to all trypanocides other than diminazine has been reported in Kenya and Somalia (Schonefeld *et al.*, 1987). Drug resistance is thus a potentially serious issue in Uganda.

### 1.6.3 Treatment of zoonotic trypanosome species

Although not yet a problem at the clinical level, recent *in vitro* studies (Matovu *et al.*, 1997) have shown a reduced sensitivity to diminazine and isometamidium of human serum resistant (*T.b. rhodesiense*) stocks isolated in the Busoga focus in Uganda. In that study, which set out specifically to test the potential usefulness of animal treatments for public health purposes, parasites were found to have a reduced sensitivity to both drugs simultaneously (multi-drug resistance). Combination therapy may therefore be necessary for animal treatments, especially in areas where animals are a reservoir of human infective trypanosomes (see White and Olliaro (1996) for a discussion of the rationale of combination therapies). Drug resistance is discussed further in Chapter VI.

### 1.7 Disease burden imposed by human sleeping sickness

#### 1.7.1 GBD study

The Global Burden of Disease study, undertaken under the sponsorship of the World Health Organization (WHO), is an attempt to assess, in an objective manner and at a global scale, the importance of different conditions that result in adverse health outcomes, with the aim of prioritising interventions. Health problems are ranked at the population level on the basis of deaths caused, termed ‘years of life lost due to premature mortality’ (YLL), and disability caused by the health problem, termed ‘years of life lived with a disability’ (YLD). The severity of the disability resulting from any particular condition is quantified, as shown in Table 1.1 (Murray, 1994;
The disability weight ascribed to sleeping sickness is 0.35 (Murray & Lopez, 1996), a Class 3 disability weight (disability resulting in a limited ability to perform certain daily activities). The YLD and YLL are combined, resulting in an estimate of the total Disability-Adjusted Life Years (DALYs), such that the population-level impact of any disease problem can be stated.

<table>
<thead>
<tr>
<th>Disability weight</th>
<th>Description of disability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.096</td>
<td>Limited ability to perform at least one activity in one of the following areas: recreation, education, procreation or occupation</td>
</tr>
<tr>
<td>0.220</td>
<td>Limited ability to perform most activities in the above 4 areas</td>
</tr>
<tr>
<td>0.400</td>
<td>Limited ability to perform activities in two or more of the above areas</td>
</tr>
<tr>
<td>0.600</td>
<td>Limited ability to perform most activities in all of the above areas</td>
</tr>
<tr>
<td>0.810</td>
<td>Needing assistance with instrumental activities of daily living such as meal preparation, shopping or housework</td>
</tr>
<tr>
<td>0.920</td>
<td>Needing assistance with activities of daily living such as eating, personal hygiene or toilet use</td>
</tr>
<tr>
<td>1</td>
<td>Death</td>
</tr>
</tbody>
</table>

Table 1.1: Disability weights and type of disability associated with each weight (Murray, 1994).

The most recent estimate of global disease burden by cause (WHO, 2001b) puts that due to sleeping sickness at 1.6 million DALYs. Sleeping sickness thus ranks approximately 80th (WHO, 1996) in comparison to the other major health problems considered, including HIV/AIDS (90 million DALYs) ischaemic heart disease (56 million), lymphatic filariasis (5.5 million), multiple sclerosis (1.5 million) and dengue fever (0.4 million).

1.7.2 T.b. rhodesiense specific DALY in Uganda

The published DALY estimates, with the disability weight given above, refer to T.b. gambiense sleeping sickness. There are more cases of T.b. gambiense than T.b. rhodesiense annually across Africa, but the burden incurred by communities affected with T.b. rhodesiense may be greater. With reference to the criteria for selection of disability weights (Table 1.1), acute sleeping sickness resulting from T.b. rhodesiense infection should be classified as a Class 5 disability (resulting in assistance being required for carrying out tasks essential to daily life), that is, a disability weight during the episode of 0.81 (Murray, 1994). With that in mind, T.b.
rhodesiense-specific DALY estimates have been calculated\(^2\) at a regional and local level using data from Uganda. When accounting for under-reporting, the sleeping sickness DALY score in the region of Uganda where this study was carried out was 161, while that for malaria in the same population was 271 (a ratio of 2:1). The ratio of the number of malaria to sleeping sickness cases was 178:1. Simply in terms of the number of cases, malaria thus appears 178 times more significant than sleeping sickness. However, when disability and death are considered (using the DALY), the relative importance of sleeping sickness is shown to be greater. There is a need, therefore, especially at the local scale, to use properly weighted measures in prioritising health interventions.

1.8 Importance of sleeping sickness research

The lack of safe drugs, compounded by the possible development of drug resistance, and the need for better diagnostic tools for treating and detecting sleeping sickness is an unfortunate reality. In terms of the epidemiology of trypanosomiasis, the fact that the disease has not been successfully controlled in the 100 years since the aetiology was first studied, is testament of the need to continue research on the subject. Maudlin et al. (in press) point out\(^3\) that although much has been written on the causes of sleeping sickness outbreaks, the subject has rarely been approached from a scientific perspective. The disease is re-emerging across Africa, especially that caused by \(T.b.\) gambiense, but also \(T.b.\) rhodesiense, which, as we have seen, has a high disease burden. In the case of \(T.b.\) rhodesiense, two processes are seen to result in this re-emergence. First, the historically recognised tendency of the disease to expand rapidly from small foci and cause epidemics. Secondly, the spread of the disease to areas previously unaffected. There is an important distinction between these processes, and it is the latter that is principally dealt with in this work.

This thesis is divided as follows. Chapters II and III present a mechanism for the establishment of a new sleeping sickness focus that has been observed in eastern Uganda, and Chapter IV provides evidence, derived from molecular studies of

\(^2\) Work carried out during the course of this PhD research.
parasites isolated in the field, for the origins of the parasites that were responsible for disease in that new focus. In the light of the evidence presented in Chapters II-IV, Chapter V analyses a set of historical data answering important questions as to the causes of the most devastating sleeping sickness epidemic recorded in East Africa at the start of the 20th Century. Chapter VI summarises the overall conclusions that can be drawn and proposes an orientation for continued sleeping sickness epidemiology studies in Uganda and more widely in East Africa.

3 Work partly carried out during the course of this PhD research.
Chapter II:
Cattle movements and
the spread of disease
2.1 Introduction

The importance of the animal reservoir of *T.b. rhodesiense* is discussed in detail in Chapter I. This Chapter deals with movements of the cattle reservoir population in Uganda, and the possible implications of those movements between 1995 and the start of the Soroti outbreak in eastern Uganda in 1999 (Fèvre *et al.*, 2001). The historical background to recent cattle movements through the region, and the methods used in the study, are discussed in this section.

2.1.1 The Karimojong and raiding

The tribal lands of the Karimojong stretch from eastern central Uganda northwards to the Sudanese border, and include Moroto and Kotido districts (see Figure 1.4). This is an area characterised by a dry savanna climate, dominated by acacia trees and open grassland (Otim, 1982). The Karimojong, a predominantly nomadic pastoral group of people of Nilo-Saharan origin, are well adapted to their environment, and have subsisted off this land for many hundreds of years. Their main subsistence production is livestock and livestock-derived products, mainly cattle, but also sheep and goats. Farming compliments this on a relatively small scale, although some sub-groups of the Karimojong practice arable farming more extensively (Emunyu, 1992). Indeed, although in the discussion that follows the term “Karimojong” will be used generically, it must be borne in mind that this is by no means a homogenous cultural group, and that different clans have quite different behavioural characteristics.

In an anthropological work following a long-term study of the Karimojong, Dyson-Hudson (1966) described in detail the relationship between Karimojong society and their animal herds. A Karimojong statement he records reads:

> “Everyone wants to find a wife, friends, happiness; to become a man of importance and influence. Without cattle, he cannot achieve any of these things. So each person thinks to himself, ‘Where shall I find cattle?’ (And the answer is) ‘From foreigners. So he resolves to go into enemy country and kill for cattle.” (p. 103).
He later states that

"...the herds of foreigners are a legitimate prize, and seizure of them does not count as theft but as enterprising self-improvement. It is laudable to kill foreigners of any age or sex..." (p. 231).

The social changes that led to armed cattle raids started during the colonial period, and continue to the present. Instability at various times has been exploited by the Karimojong, as well as other opportunistic groups on the periphery of Karamoja, resulting in large scale cattle raids. Innumerable social, ecological (Gray, 2000) and political factors continue to drive the heavily armed Karimojong (Mirzeler & Young, 2000) to raid cattle within and out with Karamoja (Emunyu, 1992; Mamdani et al., 1992; Ocan, 1992). Such raiding took place during the late 1980s and early 1990s. The cattle population in Soroti district (see Figure 1.4), where livestock farming was the basis of the economy, was reduced dramatically; estimates vary but the order of magnitude of the reduction was from over 400,000 head to approximately 12,000 (D. Mugenyi, pers. com.). That is, 97% of the animals were removed. As in other parts of sub-Saharan Africa, cattle are kept as a “living bank” in Soroti (Becker, 2000; Fèvre, 2001), sold when the need for cash arises (such as paying children’s school fees). The dramatic loss of capital had severe social consequences, and resulted in a considerable degree of emigration of the human population from Soroti at the same time.

2.1.2 Restocking

Since the return of civil stability, the human population has been resettling in Soroti and restocking has been implemented to renew the livestock population. Up-to-date data on the size of the cattle population in Soroti are, however, lacking (the last census was carried out before restocking), although one estimate (Anon., 1999) puts it at around 80,000 – that is, since re-stocking began, the cattle population may have grown by 68,000 head (660%), and is certainly still growing. Both government (Angoru, 1999; Mufumba, 1999) and NGO funds (Herbert, 1999) have been dedicated to the re-stocking process. By way of example, a UK-based NGO named
‘Send-a-Cow,’ which operates in Uganda, recently raised £241,000 in donations to fund cattle restocking in Uganda (Deedes, 2000), and has a project focussing on sustainable development targeted at women’s groups in Soroti district (Send-a-Cow, 1998). The restocking has mainly aimed at the individual farmer/family level, with a system by which offspring of donated cattle are given to neighbours within the village so that the whole community eventually benefits. This methodology is shared by several organisations involved in such work (A.S. Mubiru, pers. com.).

Wellde et al. (1989a) reported that during an outbreak of T.b. rhodesiense sleeping sickness in the Lambwe valley, Kenya, trypanosome-positive cattle that had been ear-tagged in the study area were later found some distance away, on the shores of Lake Victoria, where they were taken for trading. Eleven sleeping sickness cases occurred in the vicinity, where G. f. fuscipes is also present, and the authors concluded that, in general terms, cattle movements might have an effect on the spread of sleeping sickness. The transportation of domestic livestock across long distances, acting as reservoirs of the parasite, might thus be a potentially important issue in the causation of sleeping sickness outbreaks within tsetse infested areas. Wilde and French (1945), on studying the lack of pathology in cattle infected with human-derived trypanosomes, also came to this conclusion.

2.1.3 Hypothesis and specific aims

It is hypothesised that livestock movements linked to restocking might have been responsible for the introduction of sleeping sickness in Soroti. Here, a retrospective study was carried out to investigate whether cattle from endemic areas have been imported to Soroti as part of the re-stocking exercise. A separate, full analysis of the outbreak itself is presented in Chapter III. The outbreak in Soroti began in December 1998. In that month, 16 cases were treated in Serere Health Centre (see Chapter III). The specific aims of the work presented in this Chapter were to determine: 1) if animals have indeed been imported into Soroti as part of a re-stocking process; 2) the magnitude of the cattle movements into Soroti in the recent past; 3) the origin of the imported animals, especially with regard to the sleeping sickness status in the sites of origin; 4) how the risk of importing human infective parasites with these imported
cattle varied through time; and finally 5) to determine the theoretical potential of different control strategies to reduce the risk of importing these human infective parasites. A quantitative risk assessment (Covello & Merkhofer, 1993) was carried out to achieve these aims.

2.1.4 Risk assessments

Risk assessments are used widely in veterinary public health; for example, in assessing the risks of importing a disease to a non-affected country along with live livestock imports. Often, there is a legal requirement for such an assessment to be carried out at national and international levels. Four entire volumes of the OIE (Office International des Epizooties) scientific review have recently been devoted to “risk analysis, animal health and trade” in 1993 (Ahl et al., 1993; Kellar, 1993; MacDiarmid, 1993; Miller et al., 1993; Morley, 1993b; Morley, 1993a), “risk assessment for veterinary biologicals” in 1995 (Metcalf & McElvaine, 1995; Moos, 1995), and two volumes in 1997, “contamination of animal products: prevention and risks for animal health and for human health” (Galland, 1997; Vose, 1997).

The ultimate goal of a risk assessment procedure is to quantify risk and identify those components of the process towards which interventions could be targeted, and ultimately to implement some form of policy to reduce the risk posed by that component. There are several steps in complete risk assessment, and these have been described by Samet et al. (1998), Covello and Merkhofer (1993) and Ahl et al. (1993). The assessment is a logical process; some important definitions relating to risk are given in Table 2.1.

2.1.4.1 Stochastic or deterministic assessments

In common with other modelling applications, risk events can be described stochastically or deterministically (Anderson & May, 1992). A deterministic model does not involve chance at any stage, and is the more straightforward of the two approaches. A stochastic risk assessment takes account of uncertainty, and rather than assigning a particular fixed probability for a particular event, the probability of
an event occurring can be sampled from a range of possibilities that have been previously defined. The uncertainty inherent in the estimations is accounted for and the magnitude of the impact of the different parameters can be quantified.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard</td>
<td>Elements or events which represent potential harm</td>
</tr>
<tr>
<td>Risk</td>
<td>The likelihood of occurrence of an (adverse) event; a measure of the probability of harm</td>
</tr>
<tr>
<td>Risk assessment</td>
<td>The process of identifying and evaluating the risk of a specific hazard, in either absolute or relative terms. This process may include estimates of uncertainty</td>
</tr>
<tr>
<td>Risk analysis</td>
<td>An overall process of risk assessment, and the management of risk</td>
</tr>
</tbody>
</table>

Table 2.1: Relevant definitions relating to the study of risk. Adapted from Ahl et al. (1993).

In most cases, the assessment of risk involves breaking down an event into sequential parts; thus, the overall risk of spreading a disease through the movement of livestock from one place to another or the risk of introducing livestock pathogens into the food chain can be modelled by quantifying the chances of particular events occurring at each stage in the process. In addition, the efficacy of prevention and control interventions, such as carcass disinfection in an abattoir or screening at entry sites, can be accounted for at each stage. The overall risk in such a model is a function of the probability of individual outcomes occurring, and is dependent on the input probabilities for each stage in the process.

2.1.4.2 Case studies

Gale and Stanfield (2001) assessed the risk of transmission of BSE (Bovine Spongiform Encephalopathy) to grazing cattle and to humans eating root vegetables following the large-scale application of sewage sludge to farmland in the UK. The potential risk is derived from the washing away of prion-contaminated particulate matter from abattoirs in the sewage system. The risk of cattle contracting BSE following consumption of prion-containing soil or of humans consuming prion-contaminated soil with their root vegetables was assessed, taking into account the number of BSE-infected cows reaching abattoirs, the amount of particulate material from the cow carcasses entering the sewage system, the amount of sludge produced from sewage, the probability of the prion particles surviving sludge treatment, the
concentration of sludge on different types of farmland and the probability of the prion particles being leached in the soil (Gale & Stanfield, 2001). The model used was deterministic, in that the probability at each step was characterised by a single value, rather than being drawn from a range of possible values, and the final risk was similarly described by a single value. The risk of ingesting a prion unit from unwashed root crops was thus estimated at $1.92 \times 10^{-7}$ ID$_{50}$ prion units per person per year.

Alban et al. (2002) quantified the risk to humans of infection with *Salmonella typhimurium* DT104, a food-borne bacterial pathogen, due to consumption of cured pork sausages in Denmark. They accounted for different prevalences of bacterial contamination in locally produced and imported pork products, the effects of the preparation process (mainly fermentation) on the survival of the bacteria and finally the rate of consumption of pork sausage products in Denmark. The model used was stochastic; for example, the prevalence of bacterial infection in pork was allowed to vary for different batches of meat according to a beta distribution (Evans et al., 1993), and the reduction in the quantity of bacteria present during processing and the amount of pork sausage consumed by different age groups also varied according to appropriate distributions. The product of the different components of the model gives the final output, which was the number of potential diarrhoea cases due to *S. typhimurium* DT104 from pork sausage consumption each year. This was estimated to be between 0 and 10 cases per year, with a mode of 4 cases per year. In a stochastic model such as this, where a range of potential values are assigned to a particular event (e.g. importation of *S. typhimurium* DT104 infected pork) from a given distribution, several simulations are run, selecting, at random, a particular value for that event at each simulation from the distribution of potential values. The final output, rather than being a single output, is a range of possibilities, with confidence intervals for that range. The uncertainty inherent in the estimates of each component of the risk are thus taken into account in the analysis.

Cleaveland et al. (2002) also used a stochastic model to carry out a risk assessment for the development of rabies in humans bitten by domestic dogs in rural Tanzania.
Based on official figures, rabies is responsible for the loss of 1.11 million DALYs annually, worldwide (Fèvre et al., 2000), but this figure is thought to be an underestimate. The study in Tanzania was based on a number of disparate data sources, including the prevalence of bite injuries, the distribution of bites on the body (patients with bites near the head are at greater risk of developing clinical symptoms), the probability of developing rabies following a rabid bite, the prevalence of rabies in domestic dogs and the probability of receiving post-exposure treatment. Values for the probability of developing rabies following bites to the head, neck or arms were randomly selected from triangular distributions (Evans et al., 1993) with defined limits, while the probability of receiving post-exposure prophylaxis was assumed to be binomially distributed. The study found that, depending on the source of dog bite statistics used, the actual number of rabies deaths in Tanzania might be 10 to 100 times higher (for national bite statistics and active surveillance, respectively) than officially reported rabies deaths in the country. The risk of rabies to the rural population is thus considerable, and meaningful estimates of this risk can be made by considering, together, data acquired from different sources.
2.2 Methods

2.2.1 Cattle and human population interactions

In order to examine broad relationships between human and cattle populations, data on country-wide cattle and human populations were obtained from Rwabwoogo (1998) and the 1991 (which is the latest) Ugandan National Population and Housing Census (Government of Uganda, 1992b), respectively. Rwabwoogo (1998) is a secondary data source, presenting data supplied by the Ministry of Agriculture, Animal Industry and Fisheries, from 1993. The resolution of these data was the District level. The preferred resolution for such data is the sub-county level as this level is more relevant to the demographic pattern of the region. However, sub-county data were unavailable.

2.2.2 Cattle tracing

A retrospective study of cattle trading in a livestock market in Soroti District was undertaken between October and December 1999. The investigation was centred on the principal site involved with restocking in Soroti, Brookes Corner Market (D. Mugenyi, pers. com.). The site was chosen on the basis of discussions with the veterinary authorities locally. Kimwanga market in Mbale district was also studied as it was found to be a major supplier of livestock to Brookes Corner market.

The data were acquired from local government veterinary records collected by staff working in the market; it is a legal requirement in Uganda for the owner of any livestock animal (sheep, goats, cattle) transported to market to carry a movement permit. These permits are issued by the chief administrator at the site of origin (for example, the Local Council Chairman), and are collected by the District veterinary staff upon arrival at the market. The name of the owner, the number and type of animals transported and the point of origin are recorded. Upon leaving the market, a new movement permit is issued, and if the animal has been sold, the price paid is also recorded. Point of origin information is usually recorded to the sub-county level if the animal originated within the district; if it was sourced outwith the district.
boundary, the district of origin or the name of the market of origin was usually recorded. Animals presenting a risk of harbouring human infective trypanosomes were assumed to be those sourced within districts that are part of the traditional southeast Uganda sleeping sickness focus (Kamuli, Tororo, Iganga, Mukono and Jinja), as shown in Figure 1.4.

The records for Brookes Corner (Soroti District) and Kimwanga (Mbale District) markets were entered into a database (Microsoft Excel®). The data covered the four years leading up to the study. Data prior to this time are unavailable as a result of the civil unrest in the region. Both markets are held once a week, and market records were thus entered as weekly units and agglomerated to monthly units for the analysis. A proportion of cattle are known to be unofficially transported and sold, and these are, by definition, not included in the market data, which represent only official movements. Field veterinary staff with whom discussions were held estimated that an additional 10-25% of the market sales are traded unofficially.

2.2.3 Risk assessment

A quantitative risk assessment was carried out to estimate the risk of human infective parasites entering Soroti through Brookes Corner market, and to determine the theoretical effects, in terms of reducing this risk, of two different control options that could have been applied over the period (mass treatment or selective treatment following microscopy diagnosis). A number of parameters determine the overall risk, as represented in Figure 2.1. The risk assessment model used was based on that of Covello and Merkhofer (1993), which is summarised by Wooldridge (1996). The hazard in this study was the importation of cows from sleeping sickness endemic regions that were potentially carrying *T.b. rhodesiense*.

2.2.3.1 Data quality and parameter estimation

A stochastic model was used to estimate risk, and the risk was determined in terms of the number of animals imported per month infected with *T.b. rhodesiense*, referred to here as the *risk per month during the restocking process*. Input parameters were
drawn from various sources, as detailed below. A Monte-Carlo simulation was run in the @Risk version 4.0 software package (Palisade, Newfield, NY, USA); probability distributions were attached to each input parameter, and the model was run for 1000, 5000 and 8000 iterations. Convergence was reached between 5000 and 8000 iterations, and 8000 iterations were taken as the final output. At each iteration, the value of each input parameter was sampled at random from within the defined probability distribution for that parameter (see Table 2.2). The characteristics of the different distributions used are described by Evans et al. (1993). Of all animals traded, 12.7% (95% CI 11.5-13.9%) were from unknown areas, and were excluded from the analysis. The period under study was from April 1996 (start of the restocking) to December 1998 (start of the outbreak). Data from January 1999 onwards (restocking in Brookes Corner continued until approximately the start of 2001) were excluded. Not all the weeks during the study period are represented by the data, due to either markets not being held (such as for security reasons or closure due to foot-and-mouth quarantine), or missing data. In particular, data for the final 3 months before the outbreak started were unavailable. The estimates of the volume of importations are therefore conservative.

2.2.3.2 Monthly imports

2.2.3.2.1 Kimwanga market records

Kimwanga market in Mbale District was the major source of animals to Brookes Corner. Kimwanga is outside the sleeping sickness area; however, the data from Kimwanga market, relating to 1998, showed that on average, 37.2% (95% CI 35.7-38.7%) of the animals traded there originated in Tororo District, which does lie within the risk area. For each month, therefore, the number of animals recorded as having arrived in Brookes Corner from Kimwanga that probably originated in Tororo was determined. The probability of an animal from Kimwanga having originated in Tororo was 0.372 (binomially distributed). The monthly number imported from Kimwanga that originated in Tororo was the product of the monthly number from Kimwanga and this proportion.
2.2.3.2.2 Official imports

The total official monthly imports from risk areas (areas known to be affected by sleeping sickness) was the sum of the recorded monthly number of animals from districts affected sleeping sickness areas and the number of Kimwanga market animals that had originated in Tororo.

2.2.3.2.3 Unofficial imports

On the basis of discussions with local field staff and the District Veterinary Officer for Soroti, it was estimated that the unofficial imports to Serere from sleeping sickness areas was between 10-25% of the total imports from those risk areas. The proportion of unofficial imports in each month, and at each iteration of the model, was determined from these data, using a Beta Pert distribution, which is similar to a triangular distribution in which the mean is less dependent on the maximum and minimum values of the distribution (Palisade Corporation, 1997). The total number of unofficial imports from the risk areas was then estimated for each month, from the data on official imports, using a binomial distribution.

2.2.3.3 Proportion of T. brucei s.l. infections

Recent parasitological studies in Tororo District showed that the prevalence of T. brucei s.l. in the district was between 1-6% (Magona et al., 1998) and 0-1.4% (Magona et al., 2000), using the Buffy Coat Technique, BCT (Murray et al., 1977) for the former and the Haematocrit Centrifugation Technique, HCT (Woo, 1970) for the latter. However, a more recent study\(^4\) (unpublished obs.) has shown that microscopy has a very poor sensitivity compared to diagnosis using the Polymerase Chain Reaction (PCR); the prevalence by PCR in two livestock markets in Tororo District (Omonyole and Tuba markets) in 2001 was 28% (95% CI 19.2-36.8). The proportion of animals imported to Brookes Corner from all risk areas and estimated to be carrying T. brucei infections was therefore taken to be binomially distributed and assigned a probability of 0.28.

\(^4\) Carried out as part of this project
2.2.3.4 Proportion of T.b. rhodesiense infections

Very recent work in Soroti district, carried out as a result of the preliminary findings of this project and using a novel screening technique (see Section 2.4.2.1) show that up to 18% of all animals in the sampled areas are infected with T.b. rhodesiense (Welburn et al., 2001b). There is no evidence, however, that the prevalence of T.b. rhodesiense in Tororo was that high during the study period, nor is there evidence that it has reached those levels anywhere outside Soroti District now; the novel screening method for T.b. rhodesiense (Welburn et al., 2001b) has not yet been applied to a representative collection of bovine samples from Tororo. Therefore, the subset of T. brucei s.l. infections that are in fact human infective T.b. rhodesiense was estimated as 23%, based on RFLP results obtained from samples collected between 1988 and 1991 in Tororo (Hide et al., 1994; Hide et al., 1996).

2.2.3.5 Risk reduction

The data on importations of T.b. rhodesiense-infected cattle to Soroti through Brookes Corner were used to assess the theoretical effects of two potential interventions aimed at limiting the risk of importing these human pathogens in the reservoir host. This was done assuming that one or other of two possible treatment protocols was in place at the market, and that all animals in the market were subjected to one of the protocols. No systematic control mechanism was actually in place during the restocking process, but knowledge of the potential effectiveness of different interventions is useful for understanding the best methods for reducing the risk of importing infected animals in the future.

2.2.3.5.1 Effect of screening and selective treatment

First, the effect of screening cattle at the market by microscopy was investigated (see Figure 2.2a). Despite the objection raised above to the use of microscopy, it remains the mainstay of diagnosis at the field level. It was assumed that 100% of animals at the market were screened. Unofficial imports were assumed to be unscreened. The
sensitivity of microscopy was taken as 32.6% (95% CI 27.9-37.6), compared to the gold standard which is PCR diagnosis with standard primers (Artama et al., 1992; de Almeida et al., 1998; Kabiri et al., 1999). It was then assumed that all animals that tested positive for *T. brucei* by this method were treated with diminazine, that drug resistance was not an issue and that the efficacy of treatment was 100%. This is reasonable in Uganda where resistance to the drug has not been conclusively demonstrated in field isolates, although there is some evidence (one human-derived trypanosome stock) of decreased sensitivity of *T.b. rhodesiense* to diminazine (Matovu et al., 1997).

### 2.2.3.5.2 Effect of mass treatment

Second, the effects of mass treatment with diminazine of all cows in the market during the market day were investigated (see Figure 2.2b). Again, it was assumed that treatment with the trypanocide was 100% efficacious, and that the correct dose was always given. These treatments were only applied to officially imported animals, so some risk of parasite importation through unofficial imports would still exist.
Figure 2.1: Event tree showing risk events modelled in @Risk, leading to the importation of *T.b. rhodesiense*-infected animals to Brookes Corner. The data associated with each step are given in Table 2.2.
Figure 2.2: The hypothetical effects of two potential interventions aimed at reducing the risk of importing *T.b. rhodesiense* positive animals, that were included in the risk model (Figure 2.1). a) Microscopy diagnosis followed by treatment of trypanosome-positives for all cattle in livestock markets and b) block treatment of all animals at livestock markets. Data associated with each step are given in Table 2.2.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Distribution</th>
<th>Estimate/Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of official imports from Kimwanga originating in Tororo entering Brookes Corner each month</td>
<td>Binomial</td>
<td>n=total animals from Kimwanga market (field data) p=0.372</td>
</tr>
<tr>
<td>Number of official imports from other risk areas entering Brookes Corner each month</td>
<td>-</td>
<td>field data</td>
</tr>
<tr>
<td>Total official imports from risk areas</td>
<td>-</td>
<td>sum of official imports from Kimwanga originating in Tororo and official imports from other risk areas entering Brookes Corner</td>
</tr>
<tr>
<td>Proportion of unofficial imports originating in risk areas</td>
<td>Beta Pert</td>
<td>min=0.1, mode=0.15, max=0.25</td>
</tr>
<tr>
<td>Number of unofficial imports from risk areas entering Brookes Corner each month</td>
<td>Binomial</td>
<td>n=official imports from risk areas p= proportion of unofficial imports originating in risk areas</td>
</tr>
<tr>
<td>Proportion of risk area cattle infected with <em>T. brucei</em> spp.</td>
<td>Binomial</td>
<td>n=total imports (unofficial+official) from risk areas p=0.28</td>
</tr>
<tr>
<td>Proportion of <em>T. brucei</em> spp. that are <em>T.b. rhodesiense</em></td>
<td>Point estimate</td>
<td>0.23</td>
</tr>
<tr>
<td>Proportion of risk area cattle infected with <em>T. brusel</em></td>
<td>-</td>
<td>field data x (proportion <em>T. brucei</em> s.l. positive actually infected with <em>T.b. rhodesiense</em>)</td>
</tr>
<tr>
<td>Number of unofficial risk imports that are <em>T.b. rhodesiense</em>-positive each month</td>
<td>Binomial</td>
<td>n=total unofficial imports p=proportion of risk area cattle with <em>T.b. rhodesiense</em></td>
</tr>
<tr>
<td>Number of official risk imports that are <em>T.b. rhodesiense</em>-positive each month</td>
<td>Binomial</td>
<td>n=total official imports p=proportion of risk area cattle with <em>T.b. rhodesiense</em></td>
</tr>
<tr>
<td>Total number of <em>T.b. rhodesiense</em>-positive imports entering Brookes Corner each month</td>
<td>Binomial</td>
<td>total official <em>T.b. rhodesiense</em>-positive imports + total unofficial <em>T.b. rhodesiense</em>-positive imports</td>
</tr>
<tr>
<td>Number of <em>T.b. rhodesiense</em>-positive cows detected by microscopy</td>
<td>Binomial</td>
<td>n=official risky imports that are <em>T.b. rhodesiense</em> positive p=probability of detection by microscopy (=0.326)</td>
</tr>
</tbody>
</table>

Table 2.2: Properties of each step in the @Risk model. See text for references.
2.3 Results

2.3.1 Broad-scale relationships

Broad-scale relationships between cattle and human populations were not detected in this study; the available cattle population and cattle population density data for the traditional sleeping sickness districts in southeast Uganda (Iganga, Jinja, Kamuli, Mukono and Tororo) did not show a significant relationship with the number of sleeping sickness cases recorded ($r^2=0.006$, $p=0.9$ and $r^2=0.03$, $p=0.78$, respectively). This was also the case when the data were log-transformed. This may be the result of the low resolution of the data, which obscures relationships at more realistic biological levels. Higher resolution data for these parameters is not available in Uganda.

![Figure 2.3: Origins of cattle traded at Brookes Corner during the study period. Of those animals sourced from potential risk areas, a large proportion (36%) were sourced from Mbale district (shown in orange).](image-url)
2.3.2 Cattle tracing

The Brookes Corner market records show that, of the recorded 2796 head of cattle known to have been traded from April 1996 to December 1998, 54% (95% CI 52-55%) originated from areas where there was a potential risk of infection with T.b. rhodesiense. The origins of the traded animals are summarized in Figure 2.3. 26% (95% CI 24-27%) of the trade in cattle at Brookes corner is accounted for by local (intra-district) trade. The major sources of animals from outside Soroti are Mbale (Kimwanga market) and Tororo districts.

2.3.3 Risk assessment: disease introduction

The monthly total and cumulative total of T.b. rhodesiense-infected cattle traded in Brookes Corner market over the time period are shown in Figure 2.4. The monthly numbers of T.b. rhodesiense-positive traded cattle were highly variable, with a peak between October 1996 and June 1997, reflecting a peak in both the total number of imports and the proportion of imports from known sleeping sickness areas. The volume of livestock imports and the variations in their origins are likely to be influenced by a number of external factors such as changes in the value of livestock elsewhere in the country and the intensity of the restocking programme at any particular time. Although the socio-economic aspects of cattle trading in parts of West Africa are well understood (Josserand & Sullivan, 1979; Ariaz-Niño et al., 1980; Delgado & Staatz, 1980), a comprehensive study of the driving forces behind the cattle trade in this part of East Africa has not yet been carried out. The risk assessment shows that over the study period, between 0 and 16 T.b. rhodesiense-positive animals were imported each month. Over the entire period, a mean of 68 (95% CI 47-92 animals) T.b. rhodesiense infected animals might have been imported. As there were several months for which data were unavailable (see Section 2.2), this estimate is conservative. The estimate also assumes that the unofficial imports were always between 10 and 25% of the official importations. It is possible, however, that during months with few official imports, the proportion of unofficial imports increased, and that the risk of introducing disease during those
months was higher than estimated here (official closures of markets due to foot-and-mouth disease or other factors may have resulted in more unofficial trading).

During the months for which data are available, the mean proportion of all imported cattle (all traded cattle excluding those originating in Soroti district) that were *T. b. rhodesiense* positive per month was between 1-6% (see Figure 2.5), and may have been up to 12% in some months. Given that tsetse are abundant in the area surrounding Brookes Corner, and that the cattle that passed through the market were usually taken to local homesteads as part of organised rural development programmes (N. Machila-Eisler, pers. com.), it might have been advantageous to implement control activities in this imported cattle population during restocking. The effects this might have had are presented below.
Figure 2.4: a) Monthly and b) cumulative imports of *T.b. rhodesiense*-positive animals to Brookes Corner market. Error bars in a) show the 95% confidence intervals.
2.3.4 Risk assessment: disease control

The effects of two alternate control options were modelled. Figure 2.4b shows that a cumulative number of 68 (95% CI 47-92) *T. b. rhodesiense* infected animals passed through Brookes Corner during the study period, when no control was implemented. The first control scenario modelled in the risk analysis was microscopy plus selective treatment of positives found by this method. In this scenario, 49 (95% CI 33-69) *T. b. rhodesiense* infected animals would have entered Soroti through Brookes Corner, which is not significantly different from the number entering in the absence of any control (see Figure 2.6). This is the result of both unofficial trade that would not have been screened, but especially the poor sensitivity of microscopy, highlighting the inadequacy of field-based microscopy on bovine blood as an effective diagnostic tool during control operations.
Routine control in a market setting could also have involved block treatments; that is, trypanocidal treatment of all animals in the market. The possible effects of such an activity are also shown in Figure 2.6. Significantly fewer (11; 95% CI 4-18) *T. b. rhodesiense*-infected animals would have entered Soroti if this control intervention had been implemented in the market during the study period, compared to both no intervention and to microscopy plus selective treatment. Those that would have entered would all have been unofficially traded; unofficial trading would be difficult to intercept with any type of intervention. The benefits and problems associated with both types of intervention modelled here (mass and selective treatment) are discussed in more detail in the next section, with additional reference to other disease systems.
2.4 Discussion

The results of the work presented in this Chapter emphasise that where livestock trading is taking place with neighbouring areas that are endemic for diseases carried by livestock, there is the potential for the diseases to spread, and that efforts may be required to prevent this spread. This is especially the case with diseases such as trypanosomiasis which are of substantial public health importance (especially if, as in Serere, the local health infrastructure is not equipped to deal with the problem), but also applies to other infections such as foot-and-mouth disease, which is also a problem in eastern Uganda. A recent outbreak of foot-and-mouth disease in the UK spread quickly as a result of the large scale movements of livestock (Gibbens et al., 2001; Woolhouse & Donaldson, 2001). Uganda attempts to prevent the spread of foot-and-mouth disease within its borders by shutting down livestock markets during recognised outbreaks; it is a question of political will and financing to prioritise diseases such as sleeping sickness in the same way.

2.4.1 Importation and transmission

It is certainly possible that one local tsetse could feed on one T.b. rhodesiense-infected imported cow, and later feed on a human, resulting in clinical disease in that person. In Chapter I, the evidence favouring the reservoir-tsetse-human transmission cycle of T.b. rhodesiense over a human-fly-human cycle was discussed. In the next Chapter, the start of a major outbreak of sleeping sickness is described; in the genesis of a T.b. rhodesiense outbreak of that magnitude, with many cases reporting over a short time from different villages, the single tsetse-single cow scenario is unlikely. Rather, two possible alternate scenarios can be envisaged. The first is that a large number of infected cattle were imported to Soroti through Brookes Corner over a short time period close to the start of the outbreak, resulting in many tsetse infections. However, the risk analysis presented here has shown that the actual numbers of infected cattle that were imported to Serere through Brookes Corner was probably not very large (up to 90 over the entire period with the data available), and was spread over the entire restocking period. These were probably also widely distributed to villages around the market. The second scenario is that following the
importation of a number of infected cattle, as described by the risk analysis in this Chapter, local tsetse transmitted the parasites between the infected imports and the growing number (through restocking) of non-infected cattle. That is, the prevalence of infection in the cattle and tsetse population increased over a period of time, eventually reaching a level that made infection of a number of humans who were in contact with tsetse habitats inevitable. Eventually, the \textit{T.b. rhodesiense} prevalence in cattle reached 18%, as reported by Welburn \textit{et al.} (2001b).

With a given tsetse population, the probability of an outbreak, in simple terms, would be a product of the probability of importation of \textit{T.b. rhodesiense} in cattle and the probability of transmission by the tsetse. WHO (2001a) discusses the probability of the occurrence of a major epidemic given the introduction of a disease agent (in the context of bio-terrorism). The occurrence of an epidemic, given a disease agent and vector, is a stochastic process, partly dependent on \( R_0 \), the basic reproduction number, and \( I_0 \), the number of primary cases. Although estimates for these parameters are available (\( R_0 \) for \textit{T. brucei} was estimated to be between 0.11 and 2.54 by Rogers (Rogers, 1988)), the theoretical context proposed by WHO (2001a) applies only to much larger populations, and does not consider the outbreak situation such as that in Soroti. It would be desirable to model the probability of transmission of trypanosomes from (relatively) small populations of reservoir hosts to the human population via the vector, in order to assist in the prediction of future outbreaks in Uganda and elsewhere. Problems with such a modelling approach might include the inadequate understanding of cattle distributions and their utilisation of habitat in eastern Uganda; tsetse habitats are not uniformly distributed (detailed knowledge of tsetse distributions is particularly lacking in Soroti District and sleeping sickness free areas), and exposure of both cattle and humans to vectors and thus trypanosomes is dependent on their contact with these habitats. Tsetse, habitat and cattle interactions have been successfully studied elsewhere, including in The Gambia (Wacher \textit{et al.}, 1993; Wacher \textit{et al.}, 1994) and the Sideradougou area of Burkina Faso (de la Rocque \textit{et al.}, 1999; Michel \textit{et al.}, 1999), which resulted in zones prioritised for control efforts.
2.4.2 Interventions

Welburn et al. (2001a) have argued that controlling *T. b. rhodesiense* in the animal reservoir is the best way to reduce the incidence of sleeping sickness in the human population. They modelled the effects of different levels of chemoprophylactic coverage in cattle on the effective reduction in $R_0$ for both *T. b. rhodesiense* and *T. b. gambiense*. The $R_0$ value for *T. b. rhodesiense* was reduced by almost 100% when 100% of cattle in *T. b. rhodesiense* areas were treated, as they are the principal reservoir. This implies that mass treatment of cattle could theoretically reduce transmission of *T. b. rhodesiense* from the reservoir to the human host. In the risk analysis presented in this Chapter, mass treatment of cattle at markets was more beneficial than selective treatment following microscopy, as a large number of infected animals would have escaped detection (Figure 2.6) when using this latter method. Mass treatment of a large enough proportion of the cattle entering through the market might have reduced the risk of later transmission enough to prevent the outbreak, despite the unofficial trading that is known to occur. Woolhouse et al. (1997) tested Pareto’s Principle (Pareto, 1906), termed the 80/20-rule, in the context of the epidemiology of vector-borne and infectious diseases. This rule suggests that only 20% of the host population accounts for 80% of the value of $R_0$, and that this 20% needs to be preferentially targeted in control campaigns to reduce contact rates between infected reservoir hosts and tsetse. In the context of the importation of *T. b. rhodesiense* parasites in cattle via livestock markets, it suggests that only 20% of the cattle from risk areas might need to be treated to prevent disease importation, or at least prevent widespread transmission of the parasite.

Mass treatment is not a novel concept; mass pentamidine prophylaxis (Van Hoof et al., 1944; Waddy, 1970) has been applied in the context of *T. b. gambiense* control. Shaw et al. (1999) have shown, for schistosomiasis resulting from infection with *Schistosoma haematobium* in Senegal, that mass treatment may be a more appropriate control option in terms of reducing worm burdens and infection levels than targeted chemotherapy based on the observation of trematode eggs in urine. Targeting the most significant 20% of hosts to reduce infection rates is often practically difficult (Woolhouse et al., 1997), although in the case of both
schistosomiasis and ascariasis (caused by *Ascaris lumbricoides*), it is known that a subset of the human population at risk does indeed carry most of the infections making the targeting more straightforward. For schistosomiasis, this subset is school age children (King *et al*., 1992; Bausch & Cline, 1995), and the targeted mass treatment of this group is a cost-effective control strategy. Similarly, mass chemotherapy with levamisole was effective in reducing the prevalence of *A. lumbricoides* in a village in Nigeria, and adult infections were significantly reduced in another village by selective treatment of children 2-15 years old (Asaolu *et al*., 1991). Mass treatment has also been attempted in the context of other disease systems, with varying success. Mass prophylaxis of malaria patients in Cambodia and Thailand (Verdrager, 1986) was ineffective in reducing levels of infection with *P. falciparum* and resulted in the development of drug resistance to chloroquine. In contrast, the reduction in onchocerciasis prevalence and eventual elimination of the disease as a significant public health problem from large areas of West Africa is the result of mass chemotherapy with ivermectin combined with large-scale vector control (Boatin *et al*., 1998; Kale, 1998; Mutabazi & Duke, 1998). Sexually transmitted diseases have been controlled using targeted mass drug administration (Boily *et al*., 2000), although coverage of both low and high risk groups must remain high (over 80%), and mass treatment must reoccur often, for this to be an effective strategy. The effective reduction in the prevalence of taeniasis due to the *Taenia solium* has been shown following mass treatment of both humans and pigs with praziquantel or niclosamide (Allan *et al*., 1997; Sarti *et al*., 2000), and a 90% reduction in the prevalence and the interruption of transmission of lymphatic filariasis due to *Wuchereria bancrofti* has also been shown (Ottesen *et al*., 1997), a more effective strategy in the case of this parasite than selective chemotherapy (Simonsen *et al*., 1995).

Cattle in a market are a subset of the potentially *T.b. rhodesiense*-infected population, and mass treatment of the cattle in Brookes Corner that originated in risk areas can be equated to targeted mass chemotherapy. Whether Pareto's Principle applies to cattle infections with *T.b. rhodesiense* is not clear however, as most studies on this population of host organisms have been cross-sectional rather than
longitudinal. Longitudinal studies are ongoing in southeast Uganda (M. Eisler and I. Anderson, pers. com.), and should assist in identifying subgroups of the cattle population that might carry more infections. Torr and Mangwiro (2000) found that some cows within a cattle herd were more significant sources of blood meals to tsetse than others, a finding which was particularly associated with the age of the cattle, where older cows were fed upon more often. This older subgroup of cattle was contributing disproportionately to the maintenance of the fly population, and would thus also be a more significant potential reservoir of trypanosomes. If Pareto’s Principle does apply in this way, the development of a simple T.b. *rhodesiense* screening test (Welburn et al., 2001b) would be of additional help in specifically targeting T.b. *rhodesiense*-positive animals. Such a test, for use in field conditions, has yet to be developed (see next Section). Targeting would be beneficial in reducing the T.b. *rhodesiense* prevalence in the cattle population, but would also address the concern that mass trypanocidal treatments of entire herds might select for higher levels of drug tolerance and lead to drug resistance (Barrett, 2001).

### 2.4.2.1 SRA

*SRA*, or serum resistance associated protein, first described by de Greef et al. (1989) and de Greef and Hamers (1994), is a gene that confers resistance to human serum in *T.b. rhodesiense* parasites, making them able to survive in the human host. The insertion (transfection) of *SRA* into serum sensitive *T. b. brucei* by Xong et al. (1998), rendering them serum resistant, confirmed its role. The presence or absence of *SRA* in serum resistant (*T.b. rhodesiense*) or serum sensitive (*T. b. brucei*) parasites collected in the field was shown by Welburn et al. (2001b), using a PCR assay. Details of the mechanisms for serum resistance and lysis of trypanosomes in human hosts are discussed by Milner and Hajduk (1999). Detection of the *SRA* gene itself, or the protein for which it codes, could form the basis of a diagnostic test.

### 2.4.3 Cost-effectiveness of interventions

Although cost-effectiveness studies have recently been carried out in relation to *T.b. gambiense* control (Shaw & Cattand, 2001) and animal trypanosomiases more
generally (Kristjanson et al., 1999), similar studies on *T.b. rhodesiense* control have not been undertaken. Two major factors require consideration in assessing the cost-effectiveness of trypanosomiasis control interventions targeted at cattle. The first is whether chemotherapy of cattle is the most efficient means of control, rather than treatment of humans or tsetse trapping. Welburn et al. (2001a) have shown that this is probably the case, and cost-effectiveness analyses should thus focus on controlling the parasite in the cattle component of the transmission cycle.

The question then arises as to whether mass or targeted chemotherapy, both discussed in the previous section, is most cost-effective. The infrastructure costs of a regular microscopy screening programme are considerable, and such screening has been shown in the analysis presented here not to be very effective in reducing the numbers of imported infected cattle. For human intestinal helminth parasites, for which cost-effectiveness analyses are well developed, Bundy (1990) estimated that screening with microscopy prior to treatment increased the cost of interventions 2-6 fold, while the overall effect of mass treatment was actually greater (Evans & Guyatt, 1995). In addition, mass treatment interventions can be made even more cost-effective by targeting known groups at particularly high risk, such as children in the case of helminth infections (Evans & Guyatt, 1995), as already discussed.

The possible future development of an SRA-based screening technique for *T.b. rhodesiense* has already been suggested (Welburn et al., 2001b). Such a test could be similar to the dipstick diagnostic tools developed for field diagnosis of malaria (Mills et al., 1999; Stephens et al., 1999), and it can be assumed that using this test at the market level, to select animals for targeted treatment, would not cost more in terms of infrastructure than blanket mass treatment (similar numbers of field staff and other costs). If that were the case, it would be cost-effective to use if the combined cost of the test itself, and the cost of the doses of diminazine for the positive animals were less than the cost of treating all the animals in the market with diminazine. If it is assumed, for example, that the cost per treated cow with diminazine is US$ 0.5 (M. Eisler, pers. com.), on a market day when, for example, 100 animals are traded in the market, the cost of block treatment (manpower and
infrastructure excluded) would be US$ 50. If an SRA test at US$ 0.3 was used, the cost of screening all animals would be US$ 30 and the cost of treating 6 positives (assuming approx. 6% prevalence), would be US$ 3, coming to a total of US$ 36. It would thus be important that the cost of screening the animals be less than the cost of treating them.

Controlling human trypanosomiasis by targeting the cattle reservoir would also benefit livestock health (Fèvre et al., 2001), as other species of trypanosome would also be removed. In the absence of estimates of these overall benefits, however, livestock-targeted chemotherapy against *T. b. rhodesiense* can be assessed in terms of the reduction in human disease and the cost of this reduction in a new focus such as Soroti. Goodman et al. (1999) considered the cost-effectiveness of different malaria interventions in terms of DALYs averted, highlighting the need to rate potential interventions according to an objective outcome such as the DALY. There have been 168 sleeping sickness cases recorded so far in Serere (see Chapter III). At a cost of US$ 250 per complete treatment (WHO, 1998), the outbreak has cost the health services US$ 42,000, which does not include lost production and other social costs. If diminazine costs US$ 0.5 per treatment, 84,000 cows could have been treated for the same cost, which is the equivalent of treating every imported cow at Brookes Corner market for 53 years, at the importation rate shown by the data used in this study. Clearly, policy decisions at the national level need to consider long term costs and benefits in choosing a preferred prevention strategy.
Chapter III:
A new disease focus: establishment and expansion
3.1 Introduction

3.1.1 Background\(^5\)

3.1.1.1 Soroti district

Soroti district, which was, until 1980, part of South Teso district, lies in eastern-central Uganda, bordering Lake Kyoga (see Figure 1.4). The latest national human population census for Uganda (Government of Uganda, 1992b) counted 430,390 people in Soroti. The economy is mainly agricultural; 89% of the population live in rural areas, and the remainder in urban areas. This is reflected in the statistics for occupation, where 84.5% of the population in the district in the working age group (15 years and above) is employed in subsistence agriculture, while the national average is 70% (Government of Uganda, 1992a). This working age group is that at most risk of contracting sleeping sickness due to occupational exposure (M. Odiit, pers. com.), making the potential burden of disease heavy (see Section 1.7).

3.1.1.2 Sleeping sickness in Soroti

Sleeping sickness was identified in Soroti at Serere Health Centre\(^6\), in the southern half of the district, in December 1998 by health staff from the Livestock Research Institute (LIRI) in Tororo attending to referred patients. This was only the second time in recorded history that sleeping sickness had been seen in Soroti, which lies outside the traditional Busoga and Tororo foci in south-eastern Uganda. As a result, an active surveillance activity was undertaken during January 1999, during which 14 cases were identified over 4 days. All cases showed symptoms consistent with *Trypanosoma brucei rhodesiense* infection.

Tsetse control measures were implemented in Serere from February to July 1999, through the deployment of insecticide-treated pyramidal ‘Lancien’ traps in the areas around affected villages (Magona, pers. com.). Despite these measures, the outbreak

\(^5\) The work presented in this chapter has formed the basis of a published paper (Fèvre et al., 2001).

\(^6\) Serere Health Centre was recently designated as a Hospital (Serere Hospital), but was still a Health Centre at the time of the study, and will be referred to as such throughout.
persisted, and between 31\textsuperscript{st} December 1998 and 2\textsuperscript{nd} August 2001, a total of 168 cases were recorded. Some of the characteristics of the reported cases are shown in Figure 3.1 and Figure 3.2. There was a 1.18:1 male:female ratio in the cases. The case fatality rate for treated sleeping sickness patients has been reported as 6\% in Tororo District (Odiit et al., 1997b); all patients who presented at Serere Health Centre were treated, with a case fatality rate during the total time period in question of 4.8\%. 77\% presented with late stage symptoms.

Further studies by Odiit and colleagues (in prep) report that for each sleeping sickness case presenting at Tororo over the past 15 years, up to 15 cases do not present. That is, reporting rates among sleeping sickness patients are low, with as few as 7\% reporting to hospital. Death occurs in untreated \textit{T. b. rhodesiense} patients. There are many reasons for the low rate, including poor local knowledge of disease symptoms and reliance on traditional treatments, as well as limited access to health care facilities. These constraints are likely to be similar in Soroti, where the degree of under-reporting is almost certainly considerable.

The only reported human sleeping sickness in this part of the country prior to 1998 was a single patient found with late stage \textit{T. b. rhodesiense} in the late 1960s (Onyango, 1967). There was some doubt over the history of this case as the patient had recently travelled to and from Tanzania, a journey that would have taken her through sleeping sickness endemic areas. The case did prompt an investigation, where 650 people were examined. Apart from this one case, no parasitological evidence for infection was found, although 2.9\% of those tested had high levels of IgM in their serum samples. The observation of high IgM levels is characteristic of trypanosome infections (Omerod, 1970) as a result of the antigenic variation undertaken by the parasites, although high IgM levels may also result from a variety of other conditions (Mims, 1991) and is not strictly diagnostic. A cattle survey was also undertaken (Mwambu, 1969a; Mwambu, 1969b) showing low levels of infection with \textit{T. vivax}, but no \textit{T. brucei} s.l. were found. A survey of the fly population was also carried out following the human case report (Persoons, 1967). \textit{G. f. fuscipes} was the only fly species caught, and of 1800 flies, 0.1\% (two flies) were infected with
parasites that had pre-patent periods of between 5 and 7 days when injected into mice, which the author (Persoons, 1967) suggests is indicative of *T. brucei* s.l. infection. One of these strains was experimentally inoculated into a human, and found to be infective. No other cases have been reported from this area of Uganda since, and it has been considered sleeping sickness-free. Large tracts of the district are, however, thought to be infested with the tsetse vector *G. f. fuscipes* (J. Magona, pers. com), and Soroti lies within the fly belt (see Figure 3.6).

### 3.1.2 Hypothesis

In this chapter, it is hypothesised that the outbreak of sleeping sickness in Soroti resulted from the introduction of human infective trypanosomes to a single point source, from which they were disseminated. The sleeping sickness data are analysed using both traditional and spatial epidemiological tools, in order to test this hypothesis, and the evolving spatial dynamics of the outbreak are also investigated. A diversity of tools exist for studies of this nature, and selection of a particular methodology depends on the exact nature of the questions being asked of the data. The principal tools available are given consideration in the sections that follow.

### 3.1.3 Case control studies

Case-control studies are useful tools in epidemiology when a sample of a specific population or sub-population is analysed, and when it is necessary to have complimentary information relating to the rest of the population for comparative purposes. The diversity of uses of the case-control methodology, as well as the principal issues arising during study design, are highlighted by Schulz and Grimes (2002). Case-control studies differ from cohort studies (Grimes & Schulz, 2002) in that they are necessarily retrospective. Final outcomes, such as HIV infection or development of symptoms of asthma, are known at the start, and the proportion of individuals with (the cases) and without (the controls) the final outcome and who were all exposed to possible risk factors in the past, such as infection with herpes simplex virus (del Mar Pujades Rodriguez *et al.*, 2002) in the case of HIV, or residence in proximity to a main road in the case of asthma (Venn *et al.*, 2001), are
compared. An essential aspect of case-control study design is the avoidance of bias in the selection of controls. The controls and the cases must be derived from the same population and must share the same population characteristics other than the outcome of interest (Altman, 1997). One way of achieving this is by matching, such that the controls for any one case are drawn from a particular age range that corresponds to the age of the case. For example, in assessing the risk of intracerebral haemorrhage and the effect of the type of physical activity undertaken by patients, Thrift et al. (2002) matched cases by both age and sex. In matching, the power of the analysis can be increased by matching more than one case for each control in a one-to-many design (Woodward, 1999), although in a field situation, this many not always be possible; a one-to-one design requires extra care with the selection of controls, but properly designed studies of this nature will still yield useful findings on the aetiology of disease.

3.1.4 Spatial statistics

Point data sets are relatively easily collected and can reveal many aspects of the distribution of cases of a disease. The methods used to analyse such data sets vary considerably depending on the purpose of the analysis, the type of data involved and the characteristics of the population under study. Many examples of different sorts of analysis of point data exist in the literature, although there is often little indication of the relative merits of different tests in different contexts. A notable exception is Wartenberg and Greenberg (1993), although among their conclusions for choosing one methodology over another is 'common sense.' Many point data sets do not represent the population as a whole; that is they do not include control points. Only if control points are included can meaningful analyses be carried out.

3.1.5 The concept of spatial clustering

The identification of clusters of points within a defined geographical area may be complicated for a number of reasons. For example, many datasets are averaged over artificial administrative boundaries (such as parish- or national-level borders), whereas real world processes are governed by geographical boundaries such as
coastlines or climatic zones. These issues require consideration at both the statistical analysis and mapping stages of a study. Also of importance is the fact that a population is rarely distributed evenly in space; some areas (e.g. settlements) have a higher population density, and are therefore more likely, by chance, to see cases of a disease. Techniques are required to accommodate this (in the data collection process, it can be accounted for by the case-control methodology). For diseases that tend to affect particular age groups, confounding effects of age need to be accommodated in any analysis of clustering (Kulldorff, 1997), and these effects may also be accounted for by using a case-control design.

3.1.6 Moving window statistics

Early tools for cluster detection were able to provide likelihoods of a cluster being present or not, but were unable to identify the cluster’s location in geographic space. The former are termed ‘tests of clustering,’ and the latter ‘methods for detecting clusters,’ or ‘moving window statistics.’ The distinction between these is made by Besag and Newell (1991), and moving window statistics are reviewed by Wakefield et al. (2000).

Openshaw et al. (1987) developed a geographic analysis machine (GAM) which identified clusters in a point data set by drawing a large number of circles of fixed size and determining which circle contained the greatest number of points. This was then defined as the cluster. Turnbull et al. (1990) improved on this by fixing the circle size depending on the size of the dataset, and running a Monte-Carlo simulation to test the significance of the cluster identified. Anderson and Titterington (1997) present a similar method. The method of Turnbull et al. (1990) was further improved by Kulldorff and Nagarwalla (1995) and Kulldorff (1997), who allowed the circle size to vary during each iteration of the analysis, and therefore estimate the likelihood of a potential cluster from a theoretically infinite number of circles of different sizes. The different methodologies are fully reviewed (with reference to the supporting statistical literature) elsewhere (Kulldorff & Nagarwalla, 1995; Wakefield et al., 2000).
3.1.6.1 The Kulldorff and Nagarwalla spatial scan statistic

The spatial scan statistic (Kulldorff & Nagarwalla, 1995; Kulldorff, 1997) moves a circle systematically over the entire study area, centred at each permutation over one of the points in the dataset. The size of the circle is allowed to increase up to a defined size, ideally no more than to include 50% of the dataset in question (Kulldorff et al., 1998). For each circle size and location, the observed number of cases included in the circle is determined, and compared to the number that would have been expected under the null hypothesis. The null and alternative hypotheses are compared by maximum likelihood, as described in Kulldorff and Nagarwalla (1995). The final scan statistic output is the maximum likelihood ratio as calculated over all the different circle sizes tested. The probability, $p$, that this occurred by chance is evaluated using a Monte-Carlo simulation. This procedure randomly assigns points from the dataset as cases a pre-determined number of times, and recalculates the test statistic using this randomly generated dataset. If the value of the statistic calculated with the observed dataset ranks in the top 5% of the values calculated with the generated data, the result is considered significant. The rank of the actual statistic is the $p$-value returned (Kulldorff & Nagarwalla, 1995).

Importantly, the output of an analysis with the spatial scan statistic is a probability that clustering exists, and, if it does, that it exists in a defined location, returned as a fixed point. This point location should not be taken too literally, however; the cluster is most likely to occur somewhere within the given cluster radius around that point (Kulldorff & Nagarwalla, 1995).

3.1.6.1.1 Bernoulli or Poisson

The spatial scan statistic (Kulldorff, 1997) can be applied with one or other of two assumed distributions in a dataset, Poisson or Bernoulli. The Bernoulli model is most suitable for binary data, that is, infected or not infected (case-control type data). The Poisson model is more suitable for detecting clusters in data sets where the total population at risk is known. In such a case, the spatial unit might be the same as that in a population census (e.g. an administrative division). By either considering the
entire population (Poisson model) or using a control data set matched to the positive disease points (Bernoulli model), the scan statistic also accounts for heterogeneity in the underlying spatial distribution of the population at risk. In the case of the Bernoulli model, the "representativeness" of the control points is dependent on the stringency in the study design. It is assumed that the clusters of interest involve clusters of positive points, where being positive means being affected by a disease. Under the null hypothesis ($H_0$) in the Bernoulli model, the probability ($p$) of being positive is equal to the probability of being negative ($q$).

3.1.6.1.2 Examples: human medicine

An early application of the spatial scan statistic relates to a study of breast cancer clustering in the north-eastern United States (Kulldorff et al., 1997). Census data for the population under study was available (total population of women by county), so the Poisson model was applied. Various possible confounders, such as age, parity and race were adjusted for, and a principal cluster was identified in the New York-Philadelphia urban area. The analysis was conducted using county centroids, which resulted in a loss of resolution and a consequent inability to draw firm conclusions. In Kossi, Burkina Faso, Sankoh et al. (2001) identified particular clusters of villages that suffered from excess child mortality. This was carried out as a first step in devising and targeting population-based health improvement activities, and, using population census data and mortality surveys, spatial clustering that remained consistent over a 5-year time period was identified. The data were suitable for the Poisson model. Cousens et al. (2001) studied the distribution of cases of new-variant Creutzfeldt-Jakob (vCJD) disease in the UK. Meat consumption was weakly correlated with vCJD incidence, but, in general, their analysis of risk factors was fairly non-conclusive. However, a significant cluster of 5 cases in the Leicestershire region was detected using a Poisson model in SaTScan (the software environment developed to run the spatial scan statistic – see Section 3.2.2.3).
3.1.6.1.3 Examples: animal health studies

Work attempting to clarify the reasons for the emergence of Bovine Spongiform Encephalopathy (BSE) in the UK in the late 1980s/early 1990s (Stevenson et al., 2000a), and using parish centroids to represent the locations of affected cattle farms (in common with other studies, a central point was used to represent the smallest administrative unit), also used the spatial scan statistic. The Poisson model was used as the distribution of cases was compared to the complete cattle population in the country, and significant clustering was found in southwest England (most likely cluster), Cheshire and Cumbria (secondary and tertiary clusters respectively). Further studies went on to identify the reasons for this clustering (Stevenson et al., 2000b; Wilesmith et al., 2000), which concluded that regional differences in the methods of preparation of meat and bone meal were partly responsible. Norstrom et al. (2000) used the scan statistic (with a temporal component) to identify clustering of acute respiratory disease in Norwegian cattle, using a Bernoulli model. They identified clustering in a limited number of veterinary districts, and suggested that the clustering of the cases was indicative of an infectious origin of the disease. A general discussion of spatial analyses in animal health can be found in Pfeiffer (2000).

3.1.7 Definitions: sleeping sickness in Soroti

In the present chapter, a cluster of disease is defined as an area within the study region in which the number of disease positive points exceeds that which would be expected if the positive points were distributed in a random fashion. In other words, that more cases are occurring in particular locations than in others. This is the alternative hypothesis ($H_1$). The null hypothesis ($H_0$) is that disease positive points had an equal chance of occurring anywhere in the study region, taking into account the underlying distribution of the population (Wartenberg & Greenberg, 1993). Additionally, the interest is not only spatial, but also the temporal change in disease distribution. The null hypothesis in the case of possible temporal change is that if sleeping sickness does follow a clustered pattern, there is no change in the location of this cluster over time, whereas the alternate hypothesis can be stated such that if
sleeping sickness does follow a clustered pattern, the location of the cluster expands over time as the disease becomes established.
3.2 Methods

3.2.1 Field

A retrospective case control study was undertaken in Soroti District, eastern Uganda, in June 2000. All the cases of sleeping sickness recorded by Serere Health Centre up to this time were entered into a database. All the cases had presented from within the catchment area of the Health Centre, which extends to Serere and Kasilo counties, covering an area of 1967 sq. km. The area served by the health centre is homogenous with regard to ethnicity, the principal occupation of the inhabitants (subsistence agriculture), livestock production systems and other population characteristics.

3.2.1.1 Cases

All sleeping sickness cases were considered for inclusion, but any case lacking complete data for age, sex, month of admission or place of residence at the time of contracting the disease was automatically excluded.

3.2.1.2 Controls

Each case was matched with one control, on the basis of age, sex and month of admission to the health centre. The age classes used were <1, 1-9, 10-14, 15-19, 20-49, 50-64 and ≥65 years. The health centre inpatient, outpatient, tuberculosis, and maternity ward records were used, in that order of preference, to find a control for each case. Over 50% of controls were drawn from the inpatient records. Patients diagnosed with any vector-borne disease (including malaria) were excluded, given the possibility that similar vector habitats might bias the results. Patients were also excluded from being controls if a definitive diagnosis or details of village of residence were not reported or if they were referred from another health unit. All controls resided within the Serere Health Centre catchment area.
3.2.1.3 Georeferenced data

The basic unit of analysis was the village. Village population sizes in the study area range from under 100 to 1000 people. Village locations were recorded using a Garmin GPS+ hand-held global positioning system (GPS). Readings taken with this instrument have been accurate to within ±20 metres since the United States military removed the selective availability signal on May 2nd 2000 (Clinton, 2000). This study was conducted after that date. The data were entered in Microsoft® Excel, and geographical manipulations were conducted in the ArcView version 3.1 Geographic Information System (ESRI Systems®, Redlands, CA, USA), in which Euclidean (straight line) distances from each case and control point to Brookes Corner livestock market, were calculated.

Base layers of geographical data were obtained from the National Biomass Study of the Uganda Forestry Department (Uganda Forest Department, 1996), and geographic projections for all the collected data were transformed to conform to this dataset before any distance calculations were carried out (see Table 3.1).

<table>
<thead>
<tr>
<th>Map projection</th>
<th>Transverse mercator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spheroid</td>
<td>1880</td>
</tr>
<tr>
<td>Central meridian</td>
<td>33</td>
</tr>
<tr>
<td>Reference latitude</td>
<td>0</td>
</tr>
<tr>
<td>Scale factor</td>
<td>0.9996</td>
</tr>
<tr>
<td>False Easting</td>
<td>500,000</td>
</tr>
<tr>
<td>False Northing</td>
<td>200,000</td>
</tr>
</tbody>
</table>

Table 3.1: Projection details for the National Biomass Study dataset (Uganda Forest Department, 1996).

Distances were calculated in ArcView using the Nearest Features version 3 add-in (developed by Jeff Jenness⁷). Distances between points were calculated from centroid to centroid, and the outputs from the calculations were exported to statistical analysis software.

3.2.2 Statistical analyses

3.2.2.1 Distance varying with time

Analysis of covariance (ANCOVA), conducted in GLIM v. 4 (Crawley, 1993), was used to investigate how the distance to Brookes Corner market (km) for cases and controls varied with time since the start of the outbreak. Case or control status was defined as a categorical variable with two levels, while time (in days) since the start of the outbreak on December 31st 1998 was considered a continuous covariate. Significance of the model was tested using an F-test and the residuals were inspected for normality. This was carried out for the time period December 1998 to June 2000.

3.2.2.2 Logistic regression

The use of logistic regression for analysing case-control data is described by Lilienfeld and Stolley (1994), Woodward (1999) and Altman (1997). A conditional logistic regression was performed in S+ (MathSoft, Inc.), using the coxph command. This procedure is a variation of the Cox's Proportional Hazards model (Woodward, 1999), a regression model normally used in survival analyses (see Section 5.2.3.1). Logistic regression is appropriate for datasets with binary response variables, which in this instance are case (1) and control (0) status. Conditional logistic regression is applicable in cases, such as this, where the cases and controls are matched (Woodward, 1999). It was carried out to test whether distance from Brookes Corner market was a risk factor for sleeping sickness, and whether this changed over the time course of the outbreak. While the case-control status was used as the response variable with a unique number (1 to 113) used to identify each pair (set) of matched cases and controls, three conditional logistic regression models were fitted; distance alone (in km), time alone (in days since the first case on December 31st 1998) and the maximal model, distance and time with their interaction (distance:time interaction term). These were fitted as continuous explanatory variables. Significance was tested by comparing the reduction in deviance against the value in a $\chi^2$ table. For the
analysis, data was structured into four columns (set number, case/control status, time and distance).

3.2.2.3 Cluster detection

The spatial scan statistic (Kulldorff & Nagarwalla, 1995; Kulldorff, 1997) was used to determine the most significant spatial cluster of cases, if any, over various time periods of the outbreak. An emphasis was placed on the first 32 days of the outbreak, from the first recorded case (December 31st 1998) to the start of February 1999, prior to the 6 month-long vector control intervention, implemented around Brookes Corner market. Other times were also investigated. Analysis of clustering was performed using SaTScan\textsuperscript{8} version 2.1.3 (Kulldorff \textit{et al.}, 1998), and was run under the assumption that cases and controls followed a Bernoulli distribution (Kulldorff & Nagarwalla, 1995). In searching for clusters over different time periods, both 30- and 90- day periods were examined. The maximum cluster size was set at 50\% of the total population at risk. The analysis was run to specifically identify areas of clustering (the ‘high rates’ option) rather than areas with no clustering (Kulldorff \textit{et al.}, 1998). The analysis was consistently run with 999 Monte-Carlo iterations, which produced the same results as 9999 iterations in preliminary work, but required less computing time.

\footnote{Available from http://www.cancer.gov/prevention/bb/satscan.html}
3.3 Results

3.3.1 Descriptive statistics

Between December 1998 and the beginning of August 2001, 168 cases of confirmed sleeping sickness presented at Serere Health Centre. The number of cases reporting through time is shown in Figure 3.1. The case-control study was undertaken in June 2000, and at that time, 119 cases had reported. Of these cases, 113 were included in the case-control study. The age distribution of the 113 cases (and therefore the age-matched controls) is shown in Figure 3.2. The mean age was 29.8 years (SD 19.9 years), and 60% were male (data not shown). Six cases were excluded because no control matches were found (4 cases) or because the village of origin was unknown (2 cases). Although visual inspection of the data suggests a seasonal effect on sleeping sickness admissions, a longer time period would be required for statistical analysis of this effect.

Figure 3.1: Sleeping sickness cases in Soroti, December 1998 – August 2001. The orange section of the bar in January denotes active surveillance. There appears to be seasonality in the temporal distribution of cases (see text).
3.3.2 ANCOVA analysis

The results of the ANCOVA are shown in Figure 3.3. The minimal adequate model ($F$-ratio=131.3, df=2;223, $p<0.001$) contained significantly different intercepts ($F$-ratio=95.1, df=1;224, $p<0.001$) for the cases (3.70 km, 95% CI = 2.22-5.18 km) and controls (10.22 km, 95% CI = 8.44-12.00 km) and a positive slope for distance of cases from Brookes Corner market with respect to time since the start of the outbreak (0.014 km per day, 95% CI = 0.008-0.020 km per day, $F$-ratio=17.5, df=1;224, $p<0.001$). The slope for distance of controls from Brookes Corner against time since the start of the outbreak was not significantly different from zero and was removed from the model without any significant loss of explanatory power ($F$-value=0.45, df=1, 224, $p>0.05$).
3.3.3 Logistic regression analysis

When tested by itself using conditional logistic regression, increasing distance from Brookes Corner market was highly significantly associated with lower risk \( (\chi^2=69.30, \text{df}=1, p<0.001) \). Distance from Brookes Corner market was therefore a significant risk factor for sleeping sickness. Time since start of the outbreak (in days), when tested individually, was not significant \( (\chi^2=0.168, \text{df}=1, p>0.05) \), as expected as time was controlled for in the study design. When both continuous variables (distance and time) and their interaction were tested together, the statistical model was highly significant \( (\chi^2=81.98, \text{df}=3, p<0.001) \). This maximal model could not be simplified by the removal of the interaction term, without a significant loss of explanatory power \( (\chi^2=12.60, \text{df}=1, p<0.001) \). The parameter estimates for this
model are given in Table 1. These parameters suggest that there is no change in overall risk through time, but that the spatial distribution of risk does change with time, moving away from the market.

<table>
<thead>
<tr>
<th>Term</th>
<th>Parameter estimate</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance from Brookes Corner market</td>
<td>-0.5472 per km</td>
<td>0.0258</td>
</tr>
<tr>
<td>Time since start of outbreak</td>
<td>-0.0229 per day</td>
<td>0.1533</td>
</tr>
<tr>
<td>Distance\times time interaction</td>
<td>0.00098 per km\times day</td>
<td>0.00036</td>
</tr>
</tbody>
</table>

Table 3.2: Parameter estimates from the conditional logistic regression with two continuous variables and the interaction term. Parameter estimates are expressed as natural log odds.

### 3.3.4 Cluster detection

The detailed results of the spatial scan statistic outputs are shown in Table 3.3. During the first month of the outbreak, before any control activities were in place, a significant cluster (overall relative risk=1.93, log-likelihood ratio=30.19, p<0.001) of cases was detected (as shown in Figure 3.4). The cluster, centred on Oburiekori village, had a predicted radius of 4.2 km, and included Brookes Corner market. Twenty-seven of the 28 cases during that first time period were included in this cluster. It should be pointed out that the nature of spatial scan statistic means that the cluster centre has to be located over an existing point, and that the results therefore provide information on the most likely cluster centre, and not necessarily the true cluster centre. Cluster data for a selection of other relevant time points is shown in Figure 3.5, along with the significant cluster covering the entire time period. The details for each time point are given in Table 3.3. Although the size of the cluster changed over time, as different villages became affected by the disease and the outbreak expanded spatially, the cluster always included Brookes Corner market.
<table>
<thead>
<tr>
<th>Month</th>
<th>Villages included</th>
<th>Cases</th>
<th>Expected if random</th>
<th>RR</th>
<th>p-value</th>
<th>Cluster radius (km)</th>
<th>Log-likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>01/99</td>
<td>Oburiekori, Alilimo, Kakoke, Kangodo, Orupe</td>
<td>27</td>
<td>14</td>
<td>1.9</td>
<td>0.001</td>
<td>4.2</td>
<td>30.2</td>
</tr>
<tr>
<td>02/99</td>
<td>Igweko, Kakoke, Alilimo, Abokonyi, Agola, Pokor, Kangodo, Oburin, Akisim, Ayep</td>
<td>18</td>
<td>9</td>
<td>2.0</td>
<td>0.001</td>
<td>7.8</td>
<td>22.4</td>
</tr>
<tr>
<td>03/99</td>
<td>Adupdup, Akisim, Kyere, Ocorai, Orupe, Obur</td>
<td>6</td>
<td>3</td>
<td>2.0</td>
<td>0.008</td>
<td>15.83</td>
<td>8.3</td>
</tr>
<tr>
<td>03/00</td>
<td>Pokor, Ayep, Alilimo, Okodo, Kakoke, Orupe, Kakuja</td>
<td>11</td>
<td>6</td>
<td>1.83</td>
<td>0.001</td>
<td>9.14</td>
<td>9.75</td>
</tr>
<tr>
<td>04/00</td>
<td>Alilimo, Oteme, Okodo, Omagoro</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>0.059</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Entire period</td>
<td>Kyere, Akisim, Obar, Alilimo, Oburiekori, Obwakol, Kakoke, Kakuja, Kangodo, Ocorai, Igweko, Olupe, Kamorojo, Orupe, Ochapa, Pokor, Moru, Ojetenyang, Oteme, Oburin, Agola, Obur, Abokonyi, Omagoro, Osokotoit, Camuliki, Adupdup, Okodo, Adacar</td>
<td>99</td>
<td>56.5</td>
<td>1.75</td>
<td>0.001</td>
<td>11.04</td>
<td>72.0</td>
</tr>
</tbody>
</table>

Table 3.3: Spatial scan statistic results showing details of the identified clusters for selected months during the outbreak (total period covered is January 1999 to June 2000).
Figure 3.4: Cluster centre during the first month (January 1999) of the outbreak in Serere. The red circles are the cases, the blue squares the controls. The sizes of these points are proportional to the number of patients presenting from that village (classes for circle/square size are 1, 2-3, 4-5, 6-8, 9-13 patients).
Figure 3.5: Cluster centres (triangle) and cluster area (circle) at different time points during the study period, January 1999 to June 2000. The area covered is identical for each time period (and the area represented is approx. 2500km²), and unlike Figure 3.4, the size of the points does not represent the number of cases (for reasons of clarity). Cluster sizes are shown in Table 3.3.
3.4 Discussion

Given that domestic cattle are the principal reservoir of *T. b. rhodesiense* in Uganda (Welburn et al., 2001a), the results of this investigation on the origins of a recent sleeping sickness outbreak are highly suggestive of a cattle origin for sleeping sickness in Soroti. The outbreak is centred on Brookes Corner livestock market, where, as demonstrated in Chapter II, a significant proportion of the animals imported in the 3-4 years prior to the outbreak were potential carriers of the parasite. The location and spatial pattern of the outbreak are consistent with this conclusion.

The results show that the outbreak was initially centred close to Brookes Corner cattle market. Proximity to the market was a significant risk factor at the start of the outbreak, and although there is no change in overall risk through time, the distribution of that risk changes, moving away from the market at a rate of 1 kilometre approximately every 70 days. This suggests that sleeping sickness dispersed outwards from the initial focus during the time of the study; proximity to the market was a significant risk factor for being a case throughout, but this relationship became weaker over time. How far this dispersion will continue remains to be determined.

The clustering of cases detected in the first month, prior to the tsetse control, is consistent with tsetse acquiring infections near the market and transmitting the disease in the local area. Although the exact location of the cluster changed slightly when examined on a month by month basis, it remained centred close to Brookes Corner market through the duration of the study. The village locations (case points) in themselves are not influenced by the location of the market, and the market itself is not in a village, so the cluster centre could not fall exactly on the market itself, although it always included it. Scanning for clusters using the spatial scan statistic is based on “circular quadrats” (Kulldorff & Nagarwalla, 1995); if the distribution of case villages had been determined by a linear geographical feature (for example a river), the cluster would have included areas outside the zone of influence of that feature, that were not really part of the cluster. Although this is clearly not the case
here, it re-enforces the point that testing for clustering gives only an approximate area of increased risk because a circle is only one possible shape for a cluster. The market and the surrounding landscape lie within an area of tsetse habitat, and most of the farmers in surrounding areas who purchase animals at Brookes Corner reside in homesteads close by (N. Machilla-Eisler, pers. com.). Although there is another market within the catchment area of Serere Health Centre (Kasilo livestock market), it is over 20 km from Brookes Corner and there was no association between this market and sleeping sickness cases.

The movement of infected people into Soroti is unlikely to have resulted in the introduction of the parasite, as none of the cases of sleeping sickness at Serere Health Centre had a history of travel outside of the district. Rather, parasites were probably introduced with cattle as part of the restocking activities (see Chapter II), and were transmitted by the local tsetse. In addition, the prevalence of *T. b. rhodesiense* in cattle is much higher than that in humans (Hide *et al.*, 1996), and tsetse blood meal analysis shows that *G. f. fuscipes* is much more likely to feed on cattle than on humans (Hide *et al.*, 1996). Molecular characterisation of *T. brucei* isolates from Soroti, and their comparison to isolates from the known endemic foci follows in Chapter IV. Material for genetic studies is available from all the recorded human cases and from cattle living in the vicinity of Brookes Corner market during 2000. There was, however, no screening of cattle brought to the market itself at the start of the outbreak, as no surveillance work was ongoing at the time.

Given that the restocking activities in Uganda are being implemented on a country-wide scale as part of a long-term poverty eradication plan, it is conceivable that sleeping sickness could spread to other tsetse-infested districts of Uganda where it does not currently occur, as a result of cattle movements. The region around Lake Kyoga where this outbreak occurred, and beyond, is well endowed with riverine forest vegetation ideal for *G. f. fuscipes* (see Figure 3.6); much of this habitat became established during the times of civil unrest when the area was depopulated and left uncultivated, reverting to a bush habitat that is more suitable for the fly (no tsetse surveys were done as part of this study, however, and the contemporary distribution
of fly populations requires investigation). Since civil stability was re-established, the return of the human population has led to significant contact with this habitat and a large increase in the cattle population from restocking. The resultant interactions between humans, their livestock and the tsetse infested environment led to an increased risk of introduced parasites being transmitted. The continued spread of sleeping sickness would be expected in an area such as this, where marginal lands are being exploited. For example, Kasilo market, mentioned above, is currently involved in restocking activities, separate to those that were centred on Brookes Corner market. Initial studies (Fèvre et al., unpublished) show that animals involved in that restocking are being sourced from the Busoga sleeping sickness region, and that many animals in the market are T. brucei s.l. positive (K. Picozzi, pers. com.). The need for monitoring and further identification of parasites circulating in these animals cannot be stressed enough.

Figure 3.6: Map of Uganda showing the distribution of the major tsetse vector, G. f. fuscipes (shaded in green) and major water bodies (in blue). The red dot indicates the location of Brookes Corner livestock market. The fly distribution was estimated by LIRI entomologists in the late 1980s, and digitised from paper maps at ILRI, Nairobi.
3.4.1 The significance of the reservoir in Soroti District

During 2001, Welburn et al. (2001b) sampled 200 cattle in markets and villages within the Serere Health Centre catchment area\(^9\). Of these, 18% were reported to be positive for the \textit{SRA} gene (discussed in Section 2.4.2.1). The high prevalence of \textit{SRA} in samples from Soroti is of importance as it underlines the role played by the cattle reservoir in the transmission cycle (see Figure 1.3). With a \textit{T.b. rhodesiense} prevalence of 18% in the reservoir, there is a great need to concentrate control efforts at this component of the transmission cycle. In addition, the potential use of \textit{SRA} as a target for diagnostic tools would enable veterinary authorities to treat only those cattle which were carriers, thereby reducing the need for block treatment with trypanocides; the risk of promoting resistance to these drugs (Barrett, 2001) would thus be diminished. The general implications of these results and the need for improved intersectorial collaborations between medical and veterinary authorities are discussed in more detail in Chapter VI.

\(^9\) This work is part of the output of this PhD project.
Chapter IV:
A new disease focus:
strain typing
4.1 Introduction

Sleeping sickness has existed in Uganda for at least 100 years. The early outbreaks and epidemics were studied from a traditional epidemiological perspective, by observing patterns of disease distribution and, once their role had been established, by closely studying the distribution of the tsetse vectors (Bruce et al., 1903a). Since the advent of molecular tools and their application to the field of molecular epidemiology (Hall, 1996; Holmes, 1998; Thompson et al., 1998), the origins and dynamics of sleeping sickness outbreaks and epidemics have been studied more closely (Gibson et al., 1980; Hide et al., 1996; MacLeod et al., 2000). The early development of these tools was driven by the need to distinguish non-human infective and human infective forms of T. brucei spp. isolated from a range of hosts. The tools were essentially diagnostic, based on the presence or absence of a phenotypic characteristic. Later DNA-based tests, however, were able to distinguish parasites at the sub-specific level, and play an important part in furthering the understanding of the relationships between strains of parasite; that is, an understanding of the population structure.

The definitions of different terms applied to trypanosomes (such as ‘strain,’ and ‘isolate’) are given in Lumsden (1967) and WHO (1998). For example, an isolate is a group of viable organisms from one naturally infected host. Of particular importance in the context of the work that follows is the difference between a strain and a stock, where a strain is a group of organisms sharing a unique genetic profile (one genotype), while a stock is a population derived from the serial passage of one individual isolate. In reality, when collecting organisms from naturally infected hosts in the field, it is impossible to isolate individual strains, and the subsequent analyses carried out on field samples have always been based on stocks, which may consist of one or more strains. Indeed, Lord et al. (1999) have shown that there is probably aggregation of strains among parasite hosts. Only if individual trypanosomes are cultured and analysed would it be possible to refer to strains with confidence.
4.1.1 The available toolbox

4.1.1.1 Human serum resistance

Trypanosomes of the *T. brucei* s.l. complex are indistinguishable by microscopy. Before the advent of sophisticated molecular tools for distinguishing parasites within the complex, more basic tests based on reactions in hosts were used. Among the first examples are Heisch *et al.* (1958) and Onyango *et al.* (1966), already mentioned in Chapter I in the context of identifying non-human hosts for *T.b. rhodesiense*. In eastern Africa, parasites from animals were inoculated to human ‘volunteers,’ and the parasitological findings (that is, whether the infection became established or not) determined whether the isolate was *T. b. brucei* or *T.b. rhodesiense*. To remove the need for human experimentation, Rickman and Robson (1970) developed the Blood Incubation Infectivity Test (BIIT), in which samples were incubated in human blood *in vitro* for a period of time, following which they were inoculated into laboratory rodents. Parasites surviving this ordeal were considered human infective and therefore classed as *T.b. rhodesiense*. The technique is still used in the field, although it has been superseded by more sensitive techniques. The most significant problem with the BIIT is the need for several passages through rodents, which can result in the loss of some non-rodent adapted strains. More recently, Jenni and Brun (1982) developed the Human Serum Resistance (HSR) test, a less crude *in vitro* test using tissue culture plates based on an *in vitro* growth system (Brun *et al.*, 1981). Again, this technique involves the observation of the phenotypic characteristic of survival in human serum, to characterise stocks as either human infective or not. As such, it is unable to distinguish finer, genetic differences between stocks of trypanosome. The most notable development in diagnostic tests for differentiating *T.b. rhodesiense* from *T. b. brucei* is based on the direct detection of the *SRA* gene, and has recently been published by Welburn *et al.* (2001b) – see Section 2.4.2.1.
4.1.1.2 Isoenzymes electrophoresis

While the methods described above distinguish between human-infective and non-human-infective trypanosomes, they are unable to differentiate parasites below the sub-specific level. Isoenzyme electrophoresis was an early tool developed for this purpose. Isoenzymes are functionally identical enzymes that catalyse the same reaction but which may be structurally divergent in terms of amino acid construction (Brewer, 1970). The minor changes in the amino acid construction of an enzyme are phenotypic reflections of variation at the genotypic level, such as insertions or deletions in the sequence of base pairs in the DNA coding for the enzyme. If these enzyme variants are made up of amino acids with different molecular weights, their total molecular weights vary, and when the enzymes are placed on a starch gel (Brewer, 1970) and subjected to an electric current, they migrate through this gel at a rate depending on their molecular weight; the same principle is applied to separating DNA fragments following PCR (see Section 4.1.1.4). Isoenzyme electrophoresis was first applied to trypanosomes by Bagster and Parr (1973), and can be carried out on crude trypanosome material, where the parasitaemia has been amplified in a suitable host such as rodents or tsetse flies. Following the separation of different enzymes from this crude extract, a substrate is added which reacts with the enzyme. This reaction is detectable, and enables the products of the electrophoresis to be visualised on the gel.

Variations in isoenzyme patterns occur between stocks of trypanosomes, and quantifying the differences between isoenzyme patterns between stocks has been used as a measure of relatedness between parasites (Gibson et al., 1980). For such work, it is usually necessary to analyse variations in the electrophoretic patterns of a minimum of six enzymes. Nine commonly used isoenzymes are shown in Table 4.1. The overall enzyme profile defined for a particular population of trypanosomes relates to the pattern observed for all the enzymes used; the more enzymes used, the greater the potential variation that can be observed. Parasites that are derived from different populations but which have the same patterns are referred to as belonging to one zymodeme (Godfrey et al., 1990). Enyaru et al. (1993) analysed a large number of trypanosome isolates (151) collected from livestock, tsetse flies and humans in
south-eastern Uganda. Thirty, 12 and 24 zymodemes were detected in isolates from livestock, flies and humans respectively, demonstrating considerable variation between parasites when compared by this method. Isolates from humans showed less diversity than those from cattle and flies. Isolates from Tororo, which was a new human sleeping sickness focus at the time, were similar to those previously seen in Busoga; the Tororo focus was therefore considered an extension of the Busoga one, although the actual method of spread of human sleeping sickness into Tororo was not elucidated. Nine of the samples from domestic animals shared isoenzyme profiles with isolates from humans, a finding which confirmed that domestic livestock had an important role as reservoirs. In an earlier study with fewer isolates (Enyaru et al., 1992), no differences were detected between Tororo and Busoga isolates, although a diverse range of profiles was shown depending on the original host. In assessing the phylogenetic relatedness of the isolates from both these studies, several groupings resulted from domestic animal and tsetse fly derived isolates, while human infective parasites grouped either together or with a small group of animal or fly derived isolates – the reservoirs and vectors of human sleeping sickness.

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Abbreviated name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate protease</td>
<td>ASAT</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>ALAT</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>ICD</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>PGM</td>
</tr>
<tr>
<td>Nucleoside hydrolase</td>
<td>NHI</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH</td>
</tr>
<tr>
<td>Threonine dehydrogenase</td>
<td>TDH</td>
</tr>
<tr>
<td>Nucleoside hydrolase-deoxyinosine</td>
<td>NHD</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
</tr>
</tbody>
</table>

Table 4.1: Isoenzymes used by Hide et al. (1994)

4.1.1.3 Restriction Fragment Length Polymorphisms

Isoenzyme electrophoresis is limited in that it can detect only phenotypic variation in trypanosome strains. A more powerful technique, which involves looking for polymorphisms in restriction enzyme sites in parasite DNA, has also been used to separate and classify different trypanosome stocks. The technique is termed Restriction Fragment Length Polymorphism, or RFLP, and has yielded important information on the epidemiology of human and animal trypanosomiasis.
Briefly, trypanosome DNA is treated with a series of enzymes, which cleave the DNA molecule where certain sequences of nucleic acids occur. These enzymes are called restriction endonucleases, and different enzymes can be used that detect different nucleic acid sequences. Variation between different parasite strains will 'reside' within these restriction sites, generating a diverse number of DNA fragments of different molecular weights. These segments can be separated by running them out on an agarose gel, after which they can be transferred to a nylon filter by southern blotting. The treatment applied to these filters results in single stranded DNA bound to the filter itself, to which a complementary sequence in the form of a radio-labelled RNA probe can bind. This radio-labelled marker can be resolved on photographic paper and different banding patterns between stocks can be detected and quantified (Hide, 1996).

4.1.1.3.1 RFLP studies in Uganda

During the sleeping sickness epidemic in south-eastern Uganda, that ended in the early 1990s (Mbulamberi, 1990), a large number of samples were collected from the Tororo and Busoga region, and analysed using RFLP (Hide et al., 1990; Hide et al., 1991; Hide et al., 1994). It was found that the cattle were the most significant reservoir of human infective parasites (Hide et al., 1996) since a large number of stocks grouping with human-derived stocks were isolated from cows (group 10 in their dendrogram). 23% of all cattle carrying T. brucei spp. were carrying parasites that grouped with the stocks isolated from human sleeping sickness patients. The stocks also grouped with others isolated in previous years from Busoga and other areas around Lake Victoria, namely Busia in Kenya (Hide et al., 1991). The northern lake shore appeared from the RFLP analysis to be composed of one parasite population across isolated foci.

Earlier in this thesis (Chapter I), it was suggested that the spread of trypanosomes (and particularly human infective trypanosomes) to new areas resulting in the establishment of a focus might be driven by different epidemiological conditions than those that result in the expansion of existing foci. Hide et al. (1998) compared
the stocks of trypanosomes collected in Busoga and Tororo during the epidemic period which ended in the early 1990s with stocks collected two years later during the endemic period following that epidemic. All these stocks grouped together, indicating that the parasites involved in epidemic transmission of sleeping sickness persisted in endemic periods (although the time between the end of the epidemic and the collection of the ‘endemic stocks’ was relatively short). This suggests that if the processes resulting in spread or expansion of sleeping sickness are indeed different, then parasite factors have little impact on these processes, and other explanations, either related to cattle and human demography or environmental parameters, need to be invoked. As the parasite populations in this part of Uganda appear to be relatively stable in terms of their genetic similarities (Hide et al., 1991; Hide et al., 1996), and if the Soroti outbreak (discussed in Chapter III) is indeed derived from the Busoga/Tororo focus, it would be expected that the parasites circulating in the Soroti outbreak should have been similar to those in Tororo/Busoga.

RFLPs have yielded important data which can be used in the analysis of the relationships between trypanosome stocks, and the population structure of trypanosomes in East Africa. However, the technique requires large amounts of purified DNA, which requires culturing of parasites in vivo or in vitro. Consequently, it is cumbersome to use, and a new technique has been developed which is more rapidly and more simply carried out, and which identifies the variation between parasite strains in an equivalent way. It is Polymerase Chain Reaction (PCR)-based, and targets mobile genetic elements which naturally exist in the parasite genome.

4.1.1.4 PCR

PCR is an in vitro method of amplifying specific sequences of DNA, and was developed by Saiki et al. (1988), and is described in detail by Sambrock et al. (1989a). From a small amount of target sequence in a sample, the repetitive amplification cycles which use products from the previous cycles as a template results in a large amount of the target sequence; 20 PCR cycles result in about $1 \times 10^6$ copies of the target sequence (Roche Diagnostics, 1999). It is dependent on the use
of Taq-polymerase, a DNA-polymerase enzyme derived from a heat-stable bacterium called Thermus aquaticus (Chien et al., 1976). After running the amplified DNA on an agarose gel and staining it with appropriate methods, the presence or absence of the target in the original sequence can be determined by comparing banding patterns at the appropriate molecular weight with a control sample. In standard PCR protocols, two mirror-image oligonucleotide primers amplify the target sequence from opposite ends of each strand of DNA. PCR is sensitive and specific, and is an ideal tool for assessing the presence or absence of a particular target sequence in a sample (such as the presence or absence of the SRA gene, discussed in Chapters II and III). T. brucei s.l. consists of T. b. brucei and T.b. rhodesiense, both of which are composed of many strains reflecting the diversity of local populations of these parasites. Some of the more involved molecular epidemiological tools that are required for resolving these differences have been discussed above. The recent tool used here is the mobile-genetic-element PCR, or MGE-PCR (Hide & Tilley, 2001; Tilley, 2002).

4.1.1.5 Mobile genetic elements

The genomes of prokaryotic and eukaryotic organisms both contain sequences of DNA termed ‘transposable genetic elements’ (Shapiro, 1995). These are sequences of DNA which have the ability to move from one region of the genome to another, by either conservative transposition, where they move as complete units in a ‘cut and paste’ mechanism (Labrador & Corces, 1997), or by replicative transposition, where they remain in the original site but make copies of themselves which insert in another region of the genome (Berg & Berg, 1995). These changes occur over evolutionary time, which, due to the rapid turnover of generations in some organisms, may be short; a mobile genetic element in Drosophila spp. termed the P-element transposes four times per element per generation (Hartl et al., 1997; Hide & Tilley, 2001). By inserting into a gene, the transposable elements alter the pre-insertion sequence, possibly inactivating that gene or changing the encoded product. Drug resistance in some bacteria may be due, in part, to such a process (Arber, 2000; Carattoli, 2001; Hiramatsu et al., 2001), and other evolutionary processes may be driven by changes in genes that result from the insertion of these sequences. These
changes accumulate, making them good markers for genetic diversity, a characteristic that has been utilised for human genetic studies (Rowold & Herrera, 2000; Gagneux & Varki, 2001), and that is utilised here in the context of trypanosome populations. The type of mobile elements usually present in eukaryotic organisms such as trypanosomes and humans, are termed transposons (the other major type is termed an insertion sequence, but is important only in prokaryotes). Transposons and other types of mobile element encode the enzymes required for their own insertion into DNA (Shapiro, 1995); the natural ability of these elements to insert into the genome may make them useful in designing and producing genetically modified organisms. This has recently been proposed in relation to modifying *Anopheles gambiae*, the principal mosquito species-complex responsible for malaria transmission in sub-Saharan Africa, to render it malaria-refractory (Tu, 2001).

### 4.1.1.5.1 RIME

The major type of transposon found in trypanosome species is the retrotransposon (Aksoy, 1991). Retrotransposons insert replicatively, by producing a DNA copy of themselves via reverse transcription of an RNA intermediate (Kaiser *et al.*, 1995; Labrador & Corces, 1997). The retrotransposon targeted in the present work is called RIME (Ribosomal Mobile Element). RIME, first described by Hasan *et al.* (1984) is a useful element for detecting variation as it is both repetitive (Aksoy, 1991) and variable with respect to its position in the genome. RIME occurs several times through the genome (high copy number), and is 1028 base pairs (bp) in length. The entire RIME element is a dimer, made up of two identical halves called monomers. Each monomer, in turn, consists of two halves termed RIME A and RIME B (Hide & Tilley, 2001).

### 4.1.1.5.2 The MGE-PCR concept

Transposable elements, or Mobile Genetic Elements (MGE), can be detected by PCR. An MGE-PCR for trypanosomes has been developed by Aimee Tilley at Salford University (Tilley, 2002). The technique identifies positional variation of different copies of RIME in the whole genome. Unlike ‘traditional’ PCR, the MGE-
PCR is carried out with only a single primer, designed to begin amplification from a point within the mobile element (the end-terminal sequence) and amplify either (1) an entire sequence between elements (Figure 4.1a) or (2) the end terminal sequence itself and a small portion of the flanking region (Figure 4.1b) by mis-priming (Hide & Tilley, 2001). Primers were designed to both the RIME A and RIME B components of the monomer.

![Figure 4.1: Diagram of the amplification of mobile elements using single primers for the MGE-PCR. a) Using two elements to amplify the entire gene sequence between these elements, b) using one element and relying on mis-priming of the region flanking the element. Adapted from Hide and Tilley (2001) and Terry et al. (2001).](image)

Classically, the result of a PCR reaction would be a product of fixed length and molecular weight, since two primers that work in opposite directions amplify a highly specific region of a gene. In MGE-PCR, however, the single primer approach results in amplification of products at several regions of the genome, resulting in several products of different molecular weights (the weight of each product being dependent on the distance between different copies of the RIME element as well as mis-priming). RIME elements are often inserted in different orientations (Hasan et al., 1984), and where this occurs (see Figure 4.1), the regions between these may be amplified if the elements are close together. Electrophoresis of PCR products produces banding profiles that represent amplification of flanking regions of RIME. As the pattern of RIME element insertion varies between trypanosomes that belong to different sub-populations (that is, strains), the banding patterns vary too. The different patterns can be used to identify these different strains, and by applying phylogenetic algorithms, the variations in banding patterns between strains can be used for constructing phylogenies. MGE-PCR has already been used to successfully differentiate strains of Toxoplasma gondii in a British sheep population (Terry et al., 2001).
The MGE-PCR methodology has two main strengths. Firstly, that a large proportion of the genome can be screened for variation by the primers, reflecting the large number of mobile element insertion sites. Secondly, that prior knowledge of the flanking regions that are actually being amplified between the RIME elements is, in itself, not required in the design of the primers (Hide & Tilley, 2001). In this, MGE-PCR resembles another technique, Random Amplified Polymorphic DNA, or RAPD, but unlike RAPD (Hide, 1997), MGE-PCR primers can only amplify the region flanking a fixed point and do not anneal to different parts of the gene in a random fashion. Amplification using MGE-PCR is always anchored to the RIME element. The non-specific nature of the RAPD technique also means that it is not suitable for use with samples that are contaminated with other DNA, such as DNA from a parasite host.

4.1.2 Population structure

Three possible population structures have been proposed for trypanosome populations (Welburn et al., 2001a). Trypanosomes might show a panmictic (Tait, 1980), a clonal (Tibayrenc et al., 1990), or an epidemic population structure (Maynard-Smith et al., 1993). A panmictic population implies random mating between organisms. Such a population structure for trypanosomes was first proposed by Tait (1980), who carried out isoenzyme analyses on stocks of T. b. brucei isolated in Busoga during the late 1960s. He demonstrated single banded isoenzyme profiles as well as intermediate (hybrid) profiles, where the intermediate types were thought to show mating between the single banded types. Mating (genetic exchange) between trypanosomes has been demonstrated in laboratory adapted stocks (Jenni et al., 1986). Clonality, which implies only asexual reproduction (Tibayrenc et al., 1990) among trypanosomes, on the other hand, was proposed using previously published data on T. b. rhodesiense and other trypanosome species stocks isolated in Uganda and Kenya. Finally, the epidemic population structure (Maynard-Smith et al., 1993), in which mating occurs as a rule but in which occasionally, successful genotypes expand clonally (such as particular foci of human infective parasites), was illustrated using data derived from a number of stocks, including human, cattle and
antelope stocks from Kenya (i.e.: both T. b. brucei and T. b. rhodesiense). These different hypotheses are important in terms of understanding how trypanosome populations observed in the field relate to each other. McLeod et al. (2000) point out that the conclusions in the above studies were derived from different parasite populations, albeit originating from a similar geographical area; whereas the panmictic conclusion was drawn from a principally T. b. brucei population, the clonal conclusion was derived from analysis of T. b. rhodesiense stocks, and the epidemic conclusion from a mixture of these. Only Hide et al. (1994) looked specifically at both populations, which formed distinct groups in their analysis. T. b. rhodesiense and T. b. brucei appear to be composed of independent (and non-interbreeding) populations, possibly having evolved from a common ancestor but not currently contributing to each other’s diversity. Indeed, within the same host, they may be in competition (P.G. Coleman & S.C. Welburn, pers. com.). In summary, T. b. rhodesiense appears to be clonal, whereas T. b. brucei appears to be panmictic with the occasional expansion of an epidemic strain (MacLeod et al., 2000).

By carrying out a thorough analysis of stocks collected in Uganda in 1988 and 1990 (Hide et al., 1994) and comparing these to stocks collected in a focus in Zambia (Hide et al., 1991), it was shown that T. b. rhodesiense isolates from within a focus (e.g.: Busoga, Uganda) show little variation (consistent with clonality), but that a significant degree of variation is observed between T. b. rhodesiense isolates from different foci (e.g.: Busoga, Uganda, vs. Luangwa Valley, Zambia). This is not evidence of panmixia; rather, it supports the epidemic population structure hypothesis put forward by Maynard-Smith (1993), were clonal foci may persist for long periods of time. The geographical extent of the different foci has yet to be determined, and the hypothesis does not preclude several clones of the parasites coexisting within one focus. The limited diversity seen in T. b. rhodesiense stocks is not mirrored in T. b. brucei stocks, which show much greater diversity within limited space and time (Hide et al., 1994). Tibayrenc and Ayala (1991) state that, even if clonality is the principal mode of reproduction, it does not preclude some sexual events, and that greater diversity among T. b. brucei might simply be the result of a higher proportion of sexual activity among T. b. brucei as compared to T. b.
rhodesiense, although both might ultimately be considered clonal. Welburn et al. (2001a), however, note that an epidemic of T. b. brucei has never been observed (or indeed searched for), that natural hosts of the parasite are rarely screened outside of periods of T.b. rhodesiense epidemics, and that the population dynamics of T. b. brucei s.s. itself are simply not understood.

Although studying the population structure of trypanosomes is important in its own right, making the more practical distinction between strains of geographically and temporally separated parasites is useful in addressing the question of the origins of the Soroti district outbreak, which has been the subject of detailed epidemiological studies in Chapters II and III; it is in the context of acquiring a more complete understanding of the factors involved in causing that outbreak that MGE-PCR is used here.

4.1.3 Specific objectives

In this study, MGE-PCR was used to compare stocks of parasites isolated in Uganda, both from villages in Soroti district with active transmission of sleeping sickness and from villages with active transmission in the established Busoga and Tororo sleeping sickness foci in the southeast of the country. In both areas, parasites were collected from livestock and from humans. In addition, isolates collected at the end of the last epidemic in Tororo and Busoga were available for analysis. These stocks were compared using MGE-PCR, and the results were analysed using a phylogenetic analysis. The phylogenetic relationships were put in the context of the spatial and temporal distributions of the parasites, to address three major questions:

1. Were T.b. rhodesiense parasites in Soroti related to parasites currently circulating in the Busoga/Tororo focus in southeast Uganda?
2. Were the cattle in Soroti carrying similar T.b. rhodesiense parasites as human sleeping sickness patients in this district, suggesting that cattle might be the principal reservoir in the new focus?
3. Were the parasites currently circulating in the southeast Uganda focus similar to those isolated there over 10 years ago (Hide et al., 1991; Hide et al., 1994; Hide
et al., 1998), indicating the continued stability of the parasite population in the region?
4.2 Methods

4.2.1 Sample collection

Most of the recent trypanosome material used in the molecular analyses was collected in Uganda in May/June 2000 during a large-scale field programme that took place in four districts of the country, within the Lake Kyoga basin (see Figure 4.2). Villages that were included in the study were located in the traditional Busoga focus (Kamuli district), the Tororo focus (Tororo and Busia districts) and in Soroti district, the site of the recent outbreak discussed in Chapters II and III, which lies outside the established foci. The human samples originating in Soroti District were collected from sleeping sickness patients that had been referred to the Tororo sleeping sickness hospital during active surveillance activities in Soroti at the start of the outbreak in Serere, and the stock from a human patient originating in Kenya was also isolated at the Tororo hospital.

Figure 4.2: Area of eastern Uganda from which samples were collected. Sampled villages are shown in red.
A number of stocks collected during the early 1990s by S.C. Welburn and I. Maudlin were also analysed for comparative purposes, and these were extracted from the cryo-bank held at the Centre for Tropical Veterinary Medicine, University of Edinburgh. Details of these stocks have been published previously (Hide et al., 1994; Hide et al., 1996). The analysis was carried out blind, the identity of the stocks being revealed only once the phylogenetic analyses were complete. Details of all the recently isolated stocks used and the host from which they were derived are given in Section 4.3.

During the May/June 2000 sampling programme, three villages within each of the foci were visited, such that sampling took place in nine villages altogether. These were: Akoroi, Obar, and Obur villages (Soroti); Kinu, Bugondha and Bukafuga villages (Busoga focus) and Bugwera, Mulanga and Manakor villages (Tororo/Busia focus). Screening of the human population and of cattle and other livestock populations took place. Any trypanosome-positive human was taken to the nearest health facility for treatment, and farmers were given free treatment for any positive animal. The general health status of livestock was assessed by a veterinary team, as an additional incentive to farmers to bring their animals.

Fifty animals were screened in each village. Screening was done by two standard field methods, viz: the haematocrit centrifugation technique, HCT (Woo, 1970) and by direct dark ground/phase contrast microscopy observation of the Buffy Coat – the BCT (Murray et al., 1977; Stephen, 1986; Uilenberg, 1998). For each animal found to be T. brucei-positive by these microscopy methods, a 5 ml sample of blood was taken from the jugular vein. In Tororo/Busia and Soroti, additional blood was taken from all animals for preparation of a crude DNA sample for diagnostic PCR work.

To conform with Scottish Office importation regulations, animal blood from African countries must be passaged twice through mice prior to importation to the UK. The jugular blood sample from each T. brucei spp. positive animal was aliquoted and 0.2 ml was injected intra-peritonealy to each of two outbred Swiss white strain mice directly in the field. Sixteen percent glycerol was added to phosphate-buffered saline
with glucose (PSG) to make a 10% glycerol solution, which was added to the remaining blood sample (one part glycerol+PSG:1 part blood), which was slowly frozen in evaporating liquid nitrogen and cryo-preserved in liquid nitrogen (see Table 4.2 for recipes). Mouse parasitaemias were checked daily by observing blood extracted following a tail snip; when the parasitaemia was high (>50 parasites per field under 400× magnification or 1.96×10⁸ parasites), the mouse was anaesthetised and ex-sanguinated by cardiac puncture. The extracted blood was re-inoculated to a second mouse for each sample. When the parasitaemia in the second mouse reached ≥ 50 parasites per field, the mouse was exsanguinated as above, 10% glycerol was added (1 part glycerol+PSG:1 part blood) and the sample was slow cooled and frozen in liquid nitrogen. All double-passaged samples were transported to Edinburgh for further processing.

4.2.2 Parasite amplification

The samples of double mouse-passaged blood were defrosted and injected intra-peritoneally to two Tö strain mice per isolate that had been immuno-suppressed with cyclophosphamide. The blood was observed regularly under 400× magnification after a tail snip. Slides were read according to the method described by Herbert and Lumsden (Herbert & Lumsden, 1976). When the parasitaemia rose to approximately 1.96×10⁸ parasites/ml blood (Herbert & Lumsden, 1976), the mice were euthanised and exsanguinated by cardiac puncture. Additional stabilates of this blood were frozen and approximately 1 ml was used to extract trypanosome material.

4.2.3 Parasite extraction

A technique first described by Lanham (1968) was used to separate trypanosome material from mouse blood. The technique relies on the fact that at an appropriate pH (8.0 ± 0.05), the surface charge on the trypanosome cell surface is lower than that on the surface of red blood cells, and a suitably equilibrated column consisting of diethylaminoethyl (DEAE)-52 cellulose (Whatman®) will allow trypanosomes to
flow through with a buffer, while the red cells are retained in the matrix. In this way, a solution of trypanosomes in buffer, free of host (mouse) blood was obtained.

The DEAE-52 cellulose resin was suspended in PBS (Phosphate-Buffered Saline), and the pH adjusted appropriately (using orthophosphoric acid and/or sodium hydroxide). Fine particles were removed by repeated sedimentation. The resin was loaded into a glass funnel and allowed to settle, at which point a heparin-treated buffer (phosphate-buffered saline with glucose and heparin, or PBSGH) was allowed to run through the gel to prevent coagulation of the blood when it was loaded. Buffers were made up according to Lumsden et al. (1973), as shown in Table 4.2.

<table>
<thead>
<tr>
<th>Phosphate-Buffered Saline at pH 8.0 (PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (NaCl)</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate dihydrate (NaH₂PO₄·2H₂O)</td>
</tr>
<tr>
<td>Disodium hydrogen orthophosphate dodecahydrate (Na₂HPO₄·12H₂O)</td>
</tr>
<tr>
<td>Double distilled water</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phosphate buffered saline (pH 8.0) with glucose (PSG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose plus</td>
</tr>
<tr>
<td>PBS, made up as above</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phosphate buffered saline (pH 8.0) with glucose and heparin (PBSGH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
</tr>
<tr>
<td>Heparin</td>
</tr>
<tr>
<td>PBS, made up as above</td>
</tr>
</tbody>
</table>

Table 4.2: Recipes for solutions used in the preparation and storage of trypanosome material

One part infected mouse blood was mixed with 5 parts PBSGH buffer, and loaded on to the resin. The eluate was checked regularly for the presence of trypanosomes; when parasites were seen, the eluate was collected in a beaker packed in ice. In order to maintain flow through the resin, a head of buffer was maintained at all times. Collection was stopped when the eluate contained less than 5 parasites per field in a wet preparation at 400× magnification. The eluate was centrifuged at 2,000 rpm at 5°C for 10 minutes to pellet the trypanosomes, and the supernatant removed and
replaced with 5ml PBS to wash the parasites. This was repeated a second time and the washed trypanosome material was finally re-suspended in 5ml PBS and frozen at -20°C.

4.2.4 Concentration of parasites

Following the DEAE-cellulose purification of the 1998/2000 samples, the parasite DNA was extracted (see below); the concentration of the extracted DNA varied from 5-30 ng/ml. The lower concentration samples did not prove appropriate for the MGE-PCR, which appeared to work best with concentrations over 25 ng/ml. As a result of this, the parasite material obtained from the DEAE-cellulose columns was re-concentrated two-fold and the DNA was extracted from this re-concentrated sample.

A 1 ml sample of parasites in PBS was placed in a 1.8 ml micro-centrifuge tube, which was centrifuged at 5000 rpm for 5 minutes. This resulted in a pellet of parasite material in the tube. The supernatant was removed and 200 µl of PBS was added, and the tube and contents were vortexed for 10-15 seconds. This resulted in a 200 µl DNA sample that was five times the concentration as the original eluate from the above step.

4.2.5 DNA extraction

An aliquot (200 µl) of the trypanosome-rich solution was used in a genomic DNA extraction protocol, performed using the Qiagen® QIAamp® DNA blood minikit (Qiagen GmbH, Hilden, Germany), as per manufacturer’s instructions. The composition of the supplied solutions was not disclosed by the manufacturer. Briefly, the purified trypanosomes were lysed with 200 µl of supplied lysis buffer and in the presence of Proteinase K (20mg/ml), and the solution thoroughly mixed and incubated at 56°C for 10-30 minutes. 200 µl of 96-100% ethanol was added to the sample which was then mixed by pulse vortexing for 15 seconds. The solution was then added to the QIAamp® spin column. The column was washed firstly with
500 µl of buffer AW1 (supplied) and then again with buffer AW2 (supplied). The DNA bound to the QIAamp® spin column was eluted in AE buffer (supplied), and stored at 4°C.

The DNA concentration in each sample was measured by absorbance of electromagnetic radiation at 260 nm (A_260), using a spectrophotometer (Cecil CE 2021, Cambridge, UK) as described by Sambrock et al. (1989b). For each sample, 10ml of distilled H_2O was mixed with 1µl of the DNA solution (1:1000 dilution) in a 2.5 ml optical polystyrene cuvette (Jencons PLS, Leighton Buzzard, UK), and mixed using a dust-free mixing paddle. The absorbance at 260 nm was multiplied by 5,000 to give a DNA concentration in ng per ml.

4.2.6 DNA extraction from procyclic stocks

Samples collected in the early 1990s were held in the cryo-bank of the Centre for Tropical Veterinary Medicine, University of Edinburgh. These were procyclic forms that had been isolated from laboratory colonies of *Glossina morsitans morsitans* and grown *in vitro* (Hide et al., 1994), rather than in mice as for the samples collected as part of this study. The defrosted material (about 1 ml in each case) was centrifuged to a pellet which was re-suspended in 200ul of PBS, and the DNA was extracted as described above. These procedures were carried out by Dr Kim Picozzi at the CTVM. DNA concentrations were not measured.

4.2.7 MGE-PCR

PCR was performed at the School of Environment & Life Sciences, University of Salford, where the protocol used for this study was developed by Aimee Tilley (Hide & Tilley, 2001; Tilley, 2002). All hardware required (tubes and other consumables) was sterilised prior to use, and PCR preparations were carried out in an ultra-violet (UV) radiation cabinet to minimize the chances of contamination. PCR amplifications were performed in 50 µl reaction volumes consisting of 7 µl *Taq* DNA polymerase (HT Biotechnology Ltd, UK); 70 µl PCR buffer containing 1.5
mM MgCl (HT Biotechnology Ltd, UK); 37.5 µl sterile water (Sigma-Aldrich Inc., USA); 70 µl of primer at a concentration of 1 µM (MWG-Biotech AG, Germany); 14 µl dNTP mixture with 1 mM of each nucleotide (Bioline dNTP master mix, Bioline, USA) and 1 µl of sample DNA.

All components other than the source DNA were combined to form a master-mix in a micro-centrifuge tube which was stored on ice. Taq was added last. The source DNA was added in a separate preparation area (in order to reduce the risk of contamination). For each reaction, a negative control, consisting of all reaction ingredients minus the source DNA was also prepared. The reactions were carried out for each of three single primers (Tilley, 2002), as follows:

- RIME B (5'-AGCCATCACCGTAGAGCCCTG-3')
- REV RIME B (5'-CAGGGCTCTACGGTGATGGCT-3')
- REV RIME A (5'-ACGKTGTCCAGCAAGACGCT-3')

Amplifications were carried out using a Stratagene Robocycler 96 with a hot lid; the programme used varied with the primer, viz:

- For RIME B, the programme consisted of an initial 94°C denaturing phase for 5 minutes, followed by 35 cycles of 94°C for 50 seconds (denaturing), 55°C for 1 minute (annealing), and 72°C for 2 minutes (extension), finishing with a further 10 minutes at 72°C to ensure complete extension of any remaining fragments (Roche Diagnostics, 1999) and annealing of remaining single-stranded products,
- For REV RIME B, the programme consisted of an initial 94°C denaturing phase for 5 minutes, followed by 35 cycles of 94°C for 50 seconds (denaturing), 56°C for 1 minute (annealing), and 72°C for 2 minutes (extension), finishing with a further 10 minutes at 72°C,
- For REV RIME A, the programme consisted of an initial 94°C denaturing phase for 5 minutes, followed by 35 cycles of 94°C for 50 seconds (denaturing), 58°C
for 1 minute (annealing), and 72°C for 2 minutes (extension), finishing with a further 10 minutes at 72°C.

4.2.8 Electrophoresis

24 µl of the PCR product was added to 6 µl of bromophenol blue loading buffer and run on a 20 cm. 1.5% agarose gel in TBE buffer. The gel was stained in Ethidium Bromide (100 µl of 10mg EtBr diluted in 500 ml distilled H₂O) for 30 minutes followed by destaining in water for at least one hour. Stained gels were illuminted with a UV light source, and recorded using a Flowgen Alpha 1220 imaging system, as described in Terry et al. (2001). Gels were resolved at different exposures, which ensured that both low and high intensity bands were detected. The final banding pattern recorded for each sample was the combination of the patterns seen for that sample at the different gel exposures.

4.2.9 Measurement of banding patterns

A standard curve was constructed for each gel at each exposure using the 1 kb molecular marker which was run alongside the samples on each gel. Individual band sizes were determined by referring to this standard curve. Both the construction of the curve and the band measurements were carried out using Bio-Rad Quantity One® quantitation software version 4 (Bio-Rad, 1998), and corrected by eye. A database of all possible bands for the samples was constructed, and the presence or absence of a band of each molecular weight was recorded for every sample. The result was a profile of presence or absence of each band for each sample.

4.2.10 Phylogenetic analysis

Band presence or absence was converted to a binary code, which served as the input for the phylogenetic analysis. The “Clustering Calculator” software developed by J. Brzustowski¹⁰ was used for clustering the samples based on their similarity. The

¹⁰ Available at http://www.biology.ualberta.ca/jbrzusto/cluster.php
similarity measure used was the Jaccard Co-efficient (Jaccard, 1908), which is a simple ratio of presence or absence of a band in a stock. It is used for this type of analysis because the shared absence of a band in two samples does not imply that they are similar. That is, two stocks are considered more similar if they both have a band, than if they both lack it (Sneath & Sokal, 1973; Hide, 1996). The clustering method used was the UPGMA, or Unweighted Pair Group Method using Arithmetic averages (Sneath & Sokal, 1973), which has been used previously for phylogenetic work involving trypanosomes, and gives comparable results to other methods (Hide, 1996). Jacknifing was used as the stability analysis (these options are all standard for the software used). The output from the cluster analysis was exported to TreeView version 1.5\(^{11}\) (Page, 1996), in which dendrograms were constructed.

\(^{11}\)TreeView is available from http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
4.3 Results

4.3.1 In vivo parasite amplification

Of the 30 T. brucei-positive isolates that were collected and cryo-preserved in 1998/2000, 4 (13.3%) were not viable in the mouse amplification step in the UK. Details of the successfully amplified isolates are given in Table 4.3. All the viable isolates reached a parasitaemia between 50 and 200 parasites per field at 400× magnification (1.96×10^8 and 7.82×10^8 parasites per ml blood) measured by wet film microscopy within 6 days, enabling the mice to be exsanguinated and the DEAE-cellulose columns to be run. The variations in the parasitaemia between samples probably depended on both the initial parasitaemia of the stablate used for the inoculation and the virulence of the individual strains in a mouse host.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Original host</th>
<th>Village of origin</th>
<th>District of origin</th>
<th>Region name</th>
<th>Isolation date</th>
<th>SRA result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKO C8</td>
<td>Cow</td>
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<td>Soroti</td>
<td>Soroti</td>
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<td>-</td>
</tr>
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<td>Akoroi</td>
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<td>Soroti</td>
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<td>+</td>
</tr>
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<td>17/01/1999</td>
<td>+</td>
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<td>Soroti</td>
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<td>Soroti</td>
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<td>Soroti</td>
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<tr>
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<td>Bugwera</td>
<td>Tororo</td>
<td>Tororo</td>
<td>23/05/2000</td>
<td>-</td>
</tr>
<tr>
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<td>Cow</td>
<td>Mulanga</td>
<td>Tororo</td>
<td>Tororo</td>
<td>22/05/2000</td>
<td>+</td>
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<tr>
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<td>Kimu</td>
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<td>+</td>
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<tr>
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<td>Teso-Adungosi</td>
<td>Kenya</td>
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<td>+</td>
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Table 4.3: History of successfully purified samples collected between 1998 and 2000, and used in the MGE-PCR analysis. The history of the older stocks (1980s/1990s) has been described previously (Hide et al., 1991; Hide et al., 1994).
4.3.2 DNA concentration issues

The re-concentrated DNA samples were up to 100% more concentrated than the samples prior to re-concentration, and the banding patterns on the gels were clearer and easier to measure. It did not prove necessary to re-concentrate the stocks from 1988/1990, which were originally prepared from procyclic (tsetse salivary gland) material. The differentiation of stocks using MGE-PCR is dependent on accurate measurement of banding patterns, making clearly visible bands important. The final banding patterns used in the analysis were measured on both the pre- and post-concentration gels, which, taken together, resolved all the bands. The few samples with questionable band quality were excluded.

4.3.3 MGE-PCR gels

The MGE-PCR technique was applied using the three primers, REV A, REV B, RIME B (Tilley, 2002). However, the REV A primer did not generate enough variation between stocks and RIME B produced only faint bands that were difficult to reproduce. Conversely, REV B produced good quality bands (when the DNA concentrations were sufficient) and the results from previous RFLP analyses (Hide et al., 1994) could be reproduced using this primer alone. Therefore, although all three primers were used in the initial phases of this work, the final analysis was based only on REV B banding patterns. A representative gel, using the REV B primer, is shown in Figure 4.3. It demonstrates that an appropriate concentration of DNA is essential for accurate band measurement, especially for the lower molecular weight bands. Figure 4.4 shows samples run with higher concentrations of DNA. All the REV B PCR products were resolved on gels at different exposures, ensuring that bands of different intensities could be clearly visualised.
Figure 4.3: A typical MGE-PCR gel using the REV B primer. The samples on the left of the gel (LIRI16, AKOC20, LIRI25, LIRI26, LIRI30, LIRI31, LIRI39) were run prior to re-concentrating the DNA material, and show that the lower molecular weight bands may be difficult to identify when the concentration of DNA is low. The banding patterns are far less clear than those on the right of the gel, which were prepared from procyclic stabilates and were at a higher concentration from the outset. The extreme left hand lane is the marker (bp), the extreme right is the negative control (no sample was run in that lane). Bumanda 146 and UGL are *T. b. brucei* and *T. b. rhodesiense* (determined by the SRA PCR – see text) respectively and were run as positive controls on this gel. LIRI16-39 and AKOC20 are *T. b. rhodesiense* positive (SRA positive) stocks from Soroti. Buteba 135, Mawero 85, Papol 264, Tira 17, Tira 68 and Bumanda 25 are *T. b. brucei* (SRA negative) from Busoga/Tororo, and the remaining stocks are *T. b. rhodesiense* (SRA+), also from Busoga/Tororo. Tira 68 bands were not measured using this gel (poor profile, as shown), and Papol 264 was excluded from the analysis as lower molecular weight bands were not properly resolved, even on higher exposure gels.
Figure 4.4: MGE-PCR gel using the REV B primer. The samples are a mixture of Soroti and Tororo/Busoga samples from different time points. MULC22 is *T. b. rhodesiense* positive (determined by the *SRA* PCR), and was derived from a cow in the Tororo focus in 2000. AKOC43 and AKOC8 were collected from cows in Soroti in 2000, and are *SRA* negative (*T. b. brucei*). AKOC20 was collected from a cow in Soroti in 2000 and is *SRA* positive. UGB88 was collected from a human (*SRA+*) in Tororo in 1988/90 and Buteba 135 was collected in Tororo in 1988/90 from a cow and is *SRA* negative. The samples labelled LIR1 16-31 are human derived stocks (*SRA+*) from patients in Soroti, collected in 1998. The BIDA sample was not used in the analysis. Note that the marker increments (bp) are different to those in the previous gel. Presence or absence of bands for each sample was recorded using several gels at different exposures; for example, although MULC22 does not appear to have a band present at 823bp in the Figure, it was faintly visible on a gel at a different exposure (data not shown; see text for discussion).
4.3.4 Banding patterns and phylogenetic analysis

Table 4.4 shows the complete set of banding patterns resulting from the MGE-PCR using REV B, and the dendrogram resulting from Table 4.4 is shown in Figure 4.5. The phylogenetic analysis clearly distinguishes between those stocks that are *T.b. rhodesiense* and those that are *T. b. brucei*, as determined using the SRA PCR discussed previously (Welburn et al., 2001b). The dendrogram shows that the two sub-species are at best 78% similar when compared with this set of stocks. There is one exception (MULC22; collected in 2000), which is discussed below.

Within the *T. b. brucei* stocks, two sub-groups can be distinguished. These two groups have been labelled Group 1 and Group 2, while the *T.b. rhodesiense* group has been labelled Group 3. Among the stocks tested are several that were collected in 1988/1990 that have previously been classified using the RFLP methodology (Hide et al., 1994). With two exceptions (Tira 17 and Tira 68 – discussed below), the classification of those stocks into the *T. b. brucei* and *T.b. rhodesiense* groups is consistent using both techniques.

4.3.4.1 *T.b. rhodesiense* stocks: Group 3

4.3.4.1.1 Busoga/Tororo

KINUHBW, KINUC21, BUGH1 are human-, cow- and human-derived stocks, respectively (see Table 4.3). They are all SRA positive, and were collected in the Busoga/Tororo foci in 2000. They are identical to stocks collected in these same foci in the period 1988/1990, grouping on the same branch of the tree (100% similarity). This branch is composed of both cow and human derived stocks. The dendrogram in Figure 4.5 shows that all the *T.b. rhodesiense* stocks (other than MULC22) analysed here are >85% similar.

4.3.4.1.2 Soroti

The *T.b. rhodesiense* positive stocks collected in Soroti between 1998-2000, during the outbreak described in Chapter III, show a number of interesting characteristics.
Firstly, they are not all identical; although they all fall in Group 3 with the other *T. b. rhodesiense* stocks, there are four distinct clusters (which show >90% similarity), and within each of these clusters the stocks are >95% similar (see Figure 4.5). The first cluster is made up of AKOC20, and LIRI31; the second of LIRI26 and LIRI30; the third of LIRI37, LIRI24, LIRI39, LIRI16 and LIRI25 and the last of AKOC16 and LIRI14. Within this small sample of *T. b. rhodesiense* from Soroti (a total of 168 confirmed cases reported to Serere Health Centre up to August 2001), there are thus four possible strains of human infective parasites. Importantly, the *SRA*-positive cow-derived stocks from Soroti (AKOC20 and AKOC16) grouped within clusters of human-derived stocks. LIRI14 was collected from a patient residing in Teso District, Kenya, adjacent to Tororo (the Tororo sleeping sickness focus extends across the international border), and is very closely related (>95% similarity) to AKOC16 from Soroti, which was derived from a cow. The differences between all the *T. b. rhodesiense* stocks are explained by only 4 band differences (see Table 4.4).

### 4.3.4.2 *T. b. brucei* stocks: Group 2

As a whole, the *T. b. brucei* stocks show a great deal more diversity than the *T. b. rhodesiense* stocks, a pattern consistent with previous studies. The *T. b. brucei* stocks collected specifically in Soroti in 2000 are approximately 75% similar to each other (despite all originating in the same village). This level of dissimilarity is well within the overall pattern of diversity seen generally in the *T. b. brucei* stocks analysed.

#### 4.3.4.2.1 MULC22

The sample labelled MULC22, collected from a cow in Tororo in 2000, is the only exception to the pattern of *T. b. rhodesiense*-*T. b. brucei* clustering seen in the dendrogram (Figure 4.5). The sample tested positive for the presence of *SRA*, and has thus been classified as *T. b. rhodesiense*. Similarly to the specificity of the *SRA* PCR, certain bands on the MGE-PCR gels using the REV B primer are specific to *T. b. rhodesiense* (A. Tilley, pers. com.), although it is not known what *T. b. rhodesiense*-specific sequence is being amplified. Those bands are usually bright;
although MULC22 had this band, it was only visible faintly on a high exposure gel (data not shown). In addition, other bands resulting from the analysis of this sample are characteristic of T. b. brucei stocks. These observations suggest that the cow from which this sample was taken was carrying a mixed T.b. rhodesiense/T.b. brucei infection. If this is the case, the result is interesting for two reasons. First, that the MGE-PCR technique is unable to distinguish mixed and single infections. This is also the case with the standard T. brucei spp. diagnostic PCR and the T.b. rhodesiense-specific SRA PCR, which, by looking for presence or absence or a particular sequence, cannot show if T.b. rhodesiense is present without T. b. brucei in any one particular sample. RFLP is also unable to distinguish mixed infections. Secondly, if the anomaly in this one sample is indeed the result of a mixed infection, it suggests that mixed T.b. rhodesiense and T. b. brucei infections are rare – there are a dozen cattle-derived T.b. rhodesiense samples in the group tested, and none of those have the anomalous banding pattern shown by MULC22. In order to determine whether MULC22 is a mixed infection, it will be necessary to type stocks using MGE-PCR that have been cloned from individual parasites, obtained by serially diluting the sample. This technique is termed cloning by limiting dilution, and was described by Rosario (1981) for malaria parasites. It has been used for the kinetoplastid parasites by Handman et al. (1983), who cloned parasites from an isolate of Leishmania tropica.

4.3.4.3 T. b. brucei stocks: Group 1

The T. b. brucei in Group 1 are sufficiently different from the other T. b. brucei to merit separation into their own group (<75% similarity). The Group is made up of Tira 68, Tira 17, Papol 371 and Mela 32 which also had distinctive patterns using other techniques (Hide et al., 1994; MacLeod et al., 2000). In the RFLP analysis by Hide et al. (1994), the stocks Papol 371 and Mela 32 formed their own separate group (defined as Group 1 in that study).
|                   | 120 | 140 | 190 | 248 | 316 | 330 | 340 | 424 | 499 | 518 | 538 | 541 | 562 | 633 | 725 | 823 | 835 | 856 | 895 | 935 | 973 | 1010 | 1036 | 1080 | 1120 | 1150 | 1210 | 1350 | 1382 | 1400 | 1512 | 1550 |
|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Bumanda 25        |     |     | -   | +   | +   | +   |     |     |     |     |     | +   |     |     |     |     |     | +   |     |     |     |     |     |     |     |     |     |     |
| Buteba 135        |     |     | +   | +   | +   | -   | +   |     |     |     |     | +   | +   |     |     |     |     |     |     | +   |     |     |     |     |     |     |     |
| Fly 48            |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Fly 73            |     |     | +   | +   | +   | -   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Fly 97            |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Magola 18         |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Iyolwa 116        |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Iyolwa 125        |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Iyolwa 147        |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Katerima 116      |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Katerima 311      |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 31         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 32         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 42         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 65         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 66         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 80         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mawero 85         |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela 2            |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela 3            |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela 27           |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela 32           |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela 71           |     |     |     |     | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela Pig 1        |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Mela Pig 2        |     |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

Table 4.4: Presence or absence of bands of each size for each sample, using the REV B primer (continued over three pages)
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Figure 4.5: Dendrogram resulting from the UPGMA cluster analysis based on MGE-PCR banding patterns with the REV B primer. Three phylogenetic groupings are shown (see text). Stocks marked in red are SRA positive (T.b. rhodesiense), those in green are SRA negative (T. b. brucei). The blue rectangles highlight stocks collected in Soroti during 1998/2000.
4.4 Discussion

The molecular evidence presented in this Chapter contextualizes the sleeping sickness outbreak in Soroti District with respect to the other T.b. rhodesiense foci in Uganda (Tororo and Busoga). The evidence presented supports the hypothesis that the human infective parasites in the different foci are in fact part of the same T.b. rhodesiense population, which is distinct from the T. b. brucei population circulating in the same area.

4.4.1 T.b. rhodesiense in Soroti

Cattle in Soroti are harbouring similar stocks of human-infective parasites to those that are presently affecting the human population in the district, confirming the role of cattle as a reservoir of T.b. rhodesiense in Soroti. In addition, cattle and human T.b. rhodesiense stocks from Soroti appear to be closely related to stocks of T.b. rhodesiense presently circulating in both Tororo and Busoga, illustrated in particular by the very closely related AKOC16 stock isolated from a cow in Soroti and the LIRI014 stock isolated in Teso, Kenya, which is contiguous with the Tororo focus. As T.b. rhodesiense was not present in Soroti prior to the large scale movement of cattle into the district from the Tororo and Busoga endemic regions, it seems likely that the human infective parasites now circulating in Soroti were brought into the district from those areas with the cattle. Future studies should, however, process and analyse a larger number of samples from Soroti, to further validate this finding.

The T.b. rhodesiense stocks analysed here are diverse, but this diversity is limited; the T.b. rhodesiense parasites show >85% similarity, and the diversity among the T.b. rhodesiense group is accounted for by only 4 band differences on the MGE-PCR gels. In contrast to this limited diversity, the diversity within the T. b. brucei group is more extensive, as some of the stocks are less than 70% similar to each other. As the T.b. rhodesiense in Soroti are not all 100% identical, it can be concluded that several strains of the human infective parasite are circulating in Soroti and are responsible for the cases of sleeping sickness seen at Serere Health Centre, implying that the importation of these parasites probably occurred several times, from different
sources within Tororo and Busoga, during the restocking exercise. If the outbreak had been the result of one, very effectively transmitted imported strain, the expectation would be that all the *T. b. rhodesiense* parasites in Soroti would group very closely, with almost 100% similarity. To minimise the risk of movement of these parasites further afield, beyond Soroti, thus requires a constant monitoring effort, as suggested in Chapter II; the fact that *T. b. rhodesiense* infected animals from different parts of the established sleeping sickness affected regions were brought together in the restocking exercise emphasises the need to screen animals at a central point, such as a livestock market, rather than targeting specific areas within the affected regions.

The diversity within the small number of *T. b. rhodesiense* stocks from Soroti also illustrates that generalisations regarding strain relatedness should be made with caution when relatively small numbers of samples are examined. Certainly, a larger number of samples are needed from Soroti. However, the geographic range of many of the older samples is also limited, possibly concealing the true level of diversity that exists within *T. b. rhodesiense* from southeast Uganda. Figure 4.6 shows the distribution of cases of sleeping sickness in Tororo district between 1987 and 1997, as well as the geographical area covered by the sampling which took place during the epidemic between 1988-1990 (Hide et al., 1994; Hide et al., 1996). The area sampled had a radius of approximately 10 kilometres, and sleeping sickness in Tororo is more widely distributed than the area from which the samples came. There is thus potential diversity within the *T. b. rhodesiense* population that has not been quantified as samples from the entire range have not been analysed. Analysis of the 1988-1990 data show that even within individual villages, there is diversity. For example, in Mela village, Tororo, two *T. b. rhodesiense* stocks were collected from cattle on the same day. These are Mela2 and Mela71, and are among the most dissimilar stocks within the *T. b. rhodesiense* group (see Figure 4.5). These differences are important in interpreting the data and understanding the origins of the Soroti outbreak. Cattle imported to Soroti came mainly from Tororo and areas within Busoga. The imported cattle are likely to have had a much more diverse origin than the stocks that have been analysed, and the fact that some of the *T. b.*
rhodesiense stocks are as much as 14% dissimilar (86% similarity) might be explained by this. In short, a far greater number of samples are required from Soroti, as well as the Tororo/Busoga regions, to have a complete understanding of the population genetics of human trypanosomiasis in the region.

Figure 4.6: Outline of Tororo district, southeast Uganda. Dark blue area in the south is Lake Victoria. Areas of light blue are swamp. Sleeping sickness positive villages are shown in red (data from M. Odiit, pers. com.), Tororo town is marked by the black dot. The approximate area from which the 1988-1990 stocks were isolated (Hide et al., 1994; Hide et al., 1996) is shown by the black circle.

4.4.2 T.b. rhodesiense in Busoga/Tororo

Some of the stocks of T.b. rhodesiense recently collected in Busoga/Tororo have exactly the same banding patterns (100% similarity) as stocks isolated over a decade ago in the same region. This suggests that parts of the T.b. rhodesiense parasite population circulating in those areas are reproducing clonally (otherwise some small
degree of variation, at least, would be expected). In that respect, these data are in agreement with Hide et al. (1998), who compared parasites that had been collected during periods of both endemic and epidemic transmission. The time that had elapsed between the end of the epidemic and the so-called endemic period in that study was short (less than 5 years), allowing room for uncertainty as to the true nature of transmission (epidemic vs. endemic) at the time. However, the length of time that has now elapsed since the epidemic period is certainly significant, and the results of the present study thus provide additional support to the conclusions regarding clonality drawn by Hide and colleagues (1998). Furthermore, the continued similarity between T.b. rhodesiense parasites isolated from both cattle and from humans in the Busoga/Tororo foci confirms the important role of domestic livestock in the maintenance of the parasite during long endemic periods. T. brucei spp. are poorly pathogenic to local breeds of cattle in East Africa (Killick-Kendrick, 1971; Angus, 1996), although mild symptoms, such as a self-resolving anaemia might occasionally be seen. Therefore, T.b. rhodesiense sleeping sickness eradication efforts, if they were ever to be seriously considered, would have to concentrate on finding and treating this silent and possibly extensive reservoir of infection.

**4.4.3 T. b. brucei**

The greater degree of diversity within T. b. brucei (<70% similarity) than T.b. rhodesiense (>85% similarity) stocks suggests either that T. b. brucei parasites exchange genetic material (that is, reproduce sexually) more often than T.b. rhodesiense, or that the T. b. brucei population is also clonal but evolutionarily much older than the T.b. rhodesiense population, which would therefore have had less time for rare genetic changes to accumulate. The T. b. brucei parasites might thus fit with the ‘clonal population structure with occasional sexual events’ model, proposed by Tibayrenc and Ayala (1991). However, the apparent isolated population dynamics of the two sub-species certainly suggests that the populations of T.b. rhodesiense and T. b. brucei are quite separate and certainly do not regularly exchange genetic material. The fact that the SRA-positive trait is found only in the more tightly clustered T.b. rhodesiense group rather than being spread across both populations is further
evidence for this. These results are in agreement with the conclusions of Welburn et al. (1995), who found heterogeneities in the maturation rates of *T. b. brucei* and *T.b. rhodesiense* in both male and female tsetse flies, and suggested that this is an indicator of divergence of the two sub-species. More recently, Victoir and Dujardin (2002) suggested that sexual recombination events may not be necessary to explain diversity among strains of parasites, basing this on observations made on *Leishmania* spp., and the variable organisation of *gp63* genes (coding for a surface glycoprotein).

Parasites may have evolved methods of generating diversity in their own genomes by self-regulated genome re-organisation without reverting to sexual events. The limited diversity seen in *T.b. rhodesiense* stocks (compared to *T. b. brucei*) might be accounted for by such a mechanism.

### 4.4.3.1 Prior occurrence of *T. brucei* in cattle in Soroti

The epidemiological evidence for the recent importation of *T.b. rhodesiense* to Soroti District has been discussed in Chapters II and III. As discussed in Chapter III, one case had been previously identified there in the 1960s, but the case history suggested that the infection had been acquired elsewhere. Similarly, there is little published evidence of the occurrence of *T. b. brucei* in the district. Mwambu (1969b) sampled 2444 cattle in Serere and other parts of Soroti, and found, using a combination of microscopy and the IFAT (Indirect Fluorescent Antibody Technique) that only *T. vivax* was prevalent (57.5% prevalence – as this is an antibody test, it includes cured as well as current infections). Ford (1971) cites Ugandan veterinary reports from the period 1949-1955, showing a *T. brucei* prevalence of 0.12% (7 of 5967 positive slides). Unfortunately, the data for recent cross-sectional surveys of cattle in different parts of Soroti are not yet available, but early results show a high *T. brucei* spp. prevalence. Those data refer to the period after the start of the sleeping sickness outbreak in Soroti, and reliable prevalence information for the period prior to the introduction of sleeping sickness will remain unavailable. Whether *T. b. brucei* has previously been widespread in Soroti or not, the three stocks of *T. b. brucei* isolated in the district as part of this study are both distinct from each other while also showing some similarities to other *T. b. brucei* parasites from the Tororo/Busoga foci (though none are identical). If it is the case that *T. b. brucei* was not widespread here
prior to the importation of sleeping sickness, the diversity seen among the *T. b. brucei* in Soroti adds weight to the suggestion that many parasite strains were imported to the District at different times during restocking. If those strains of *T. b. brucei* were present in Soroti prior to the outbreak, it suggests that the *T. b. brucei* in the whole of eastern Uganda make up one intermixing population.

### 4.4.3.2 *T. b. brucei*: Group 1

Tira 17 and Tira 68, which are *SRA*-negative and which have been classified as Group 1 in this study, were grouped with the *T.b. rhodesiense* parasites using RFLP (Hide *et al.*, 1994). However, McLeod *et al.* (2000), using minisatellite markers, contradicted this finding and grouped them as *T. b. brucei*, a finding which is in agreement with the results of this study. Further work on these stocks, particularly in elucidating the reasons for their classification as *T.b. rhodesiense* using RFLP, is required, but is beyond the scope of this project.

### 4.4.4 Concluding remarks

The following conclusions can be drawn as a result of the analysis conducted during this study:

- Cattle in Soroti are carrying *T.b. rhodesiense* strains similar to those affecting humans in the district, suggesting that cattle are a reservoir of human infective parasites in Soroti.
- Cattle in Soroti are carrying strains almost identical to stocks isolated in the Tororo/Busoga focus, which is consistent with the hypothesis that cattle imported from sleeping sickness regions in Tororo and Busoga during restocking carried *T.b. rhodesiense* to Soroti.
- More than one *T.b. rhodesiense* strain is present in Soroti, indicating that the human infective parasite was probably imported on more than one occasion.
- Some *T.b. rhodesiense* strains collected over 10 years apart from Busoga/Tororo are identical, a characteristic of a clonally reproducing population (no genetic exchange).
- Domestic animals are maintaining parasites in the stable, endemic foci of Tororo and Busoga.
- T. b. brucei is diverse, and does not appear to exchange genetic material with T.b. rhodesiense. The level of diversity among T. b. brucei is suggestive that at a population level, more genetic exchange occurs in this sub-species than in T.b. rhodesiense.
- Restocking may have also been responsible for an increased prevalence of T. b. brucei in Soroti.

The phylogenetic similarity of the stocks in Soroti and Tororo/Busoga, from both the cattle and the human population, when taken together with the evidence from the field studies presented in Chapters II and III, suggests that the establishment of the focus in Soroti is a direct result of the importation of T.b. rhodesiense-infected cattle from Tororo and Busoga to Soroti, and that, more generally, cattle movements are the driving force for the spread of sleeping sickness in regions affected by the zoonotic form of trypanosomiasis. It is likely that the epidemic in Tororo in the 1980s/1990s was also instigated by similar livestock-related factors, although the investigations of disease spread at the time concentrated on the fly component of the transmission cycle, despite the fact that the significance of the cattle reservoir had been established (Hide et al., 1994; Hide et al., 1996).

In terms of the expansion of existing foci during occasional epidemic periods (as distinct from disease spread which has been investigated here), there is no doubt that entomological factors, such as the rapid expansion of fly habitats as a result of environmental changes, play an important role (Mbulamberi, 1989; Wellde et al., 1989b; Lancien, 1991). However, for these epidemics to be initiated, the human infective parasites must be surviving unnoticed in the foci outside of the epidemic periods. As T. brucei spp. are not pathogenic to local East African cattle breeds such as Zebu, and as T.b. rhodesiense appears to persist clonally, this is not difficult to envisage. In this situation, the occasional chance infection of flies would result in occasional human sleeping sickness cases, as is observed during endemic periods. There is a need to further understand the detailed behaviour and biology of cattle
populations, especially with regard to the way in which livestock, humans, tsetse and the environment interact, in order to achieve accurate predictions of the timing and nature of the expansion of existing foci.
Chapter V:
The 1900-1910 epidemic revisited
5.1 Introduction

5.1.1 Epidemic sleeping sickness in Uganda: 1901

At the start of the 20th century, Uganda was affected by a large scale and disastrous epidemic of sleeping sickness. It is estimated that around 250,000 people died (Langlands, 1967) during the period 1900-1920 in the Busoga and Buganda regions of the country (the Buganda region extends west of Busoga – see Figure 1.4). The first published description of sleeping sickness cases was made by the Cook brothers, who were medical missionaries with the CMS - Church Missionary Society (Cook, 1901; Cook, 1902), and who had set up a mission hospital. The extent of the epidemic became clear as the number of cases seen in their hospital and other medical facilities increased, and as the disease was identified in many areas around the northern shore of Lake Victoria (Bruce et al., 1903b; Christy, 1903).

The study of sleeping sickness was initially focussed on discovering the causative agent. As well as being newly recognised in Uganda, the disease had not been previously described in eastern Africa as a whole. Indeed, the discovery of trypanosomes as a disease agent was relatively recent. The causative agent of ‘Trypanosoma fever’ in the Gambia, West Africa was noticed in 1902 by Forde (1902) and described by Dutton (1902) as *T. gambiense*. Manson (1898) was of the belief that the disease was linked to *Filaria perstans* (now known as *Mansonella perstans*), a blood-dwelling nematode that is of no clinical importance. Manson had limited evidence to support his claim (Cook, 1901; Foster, 1970), which may have been promulgated, in part, for political motivations (Haynes, 2000). None-the-less, before the causative link to trypanosomes was made, early diagnostic efforts in Uganda and elsewhere on sleeping sickness patients focussed on finding this organism. *T.b. rhodesiense* was only described (in present day Zambia) in 1910 (Stephens & Fantham, 1910), and the importance of *T.b. rhodesiense* in Uganda was only confirmed during the next major epidemic to affect the country 40 years later (MacKichan, 1944).
In 1903, both Castellani (1903) and Bruce et al. (1903b) found an association between the occurrence of trypanosomes and sleeping sickness in Uganda. From this point onwards, the parasitological assessment of suspect sleeping sickness cases was based on the observation of trypanosomes in blood or cerebro-spinal fluid. There followed, in Uganda, much work on the distribution of the disease and the distribution of various potential vectors. Bruce et al. (1903a) found that the geographical extent of sleeping sickness corresponded with the distribution of the tsetse fly, work which followed earlier observations in Zululand (1895) on the vector responsible for a cattle disease termed nagana (cattle trypanosomiasis).

5.1.2 A new disease?

It is not known how long sleeping sickness had existed in Uganda prior to the epidemic. Most of the pioneering scientists seem to have assumed it was a new problem as they found little or no evidence for it having occurred there before. The assumption was made, however, that such an epidemic disease would have always occurred in the same epidemic proportions that were being observed. However, Christy (1903), while travelling through southern Uganda, noted that sleeping sickness had probably been present long before it was first discovered around 1901, and that it probably originated in Busoga, the core of the present day T.b. rhodesiense focus, and Duke (1918), on reviewing the available evidence, also states that ‘some form of human trypanosomiasis’ had been present around the Ugandan shores of Lake Victoria prior to the epidemic.

5.1.2.1 The causative organism

Confusion over the novelty or otherwise of sleeping sickness in this area was intertwined with the available knowledge of the causative organism. During the epidemic starting in 1901, only one species of human infective trypanosome had been identified, to which the epidemic in Uganda was ascribed. This was the T. gambiense parasite that had been observed in the Gambia (Dutton, 1902). Furthermore, concurrently with the epidemic in Uganda, sleeping sickness was recognised in many parts of central Africa (Morris, 1963). It had not been seen
previously elsewhere in eastern Africa, and the understandable assumption was made that the Ugandan disease was derived from the sleeping sickness epidemic raging westwards in the (present day) Democratic Republic of Congo and elsewhere.

If sleeping sickness at the time had been due to *T. b. gambiense*, it would suggest, as some have (Morris, 1963), that the parasite was imported from the west as part of large scale human population movements that are known to have occurred at the time. Only Köerner (1994) and Köerner *et al.* (1995) have seriously questioned the identity of the parasite responsible for the first Ugandan epidemic. They argue that as *T. b. rhodesiense* occurs in apparently stable endemic foci which occasionally expand resulting in epidemics, that *T. b. rhodesiense* was probably present in Busoga long before 1901. This is an attractive argument, as the wholesale replacement of one parasite species by another (*T. b. gambiense* by *T. b. rhodesiense*) in a region seems unlikely.

While working in Uganda at the start of the epidemic, Castellani (1903) noticed two distinct clinical symptoms among the patients presenting. The first he called Trypanosoma fever, as is was similar to the disease seen in The Gambia and ascribed to *T. gambiense*. The second he called sleeping sickness, and tentatively called the trypanosome that he found in those cases *T. ugandense* (Castellani, 1903). The distinction was essentially clinical; what he called sleeping sickness was a much more virulent infection than the Trypanosoma fever caused by *T. gambiense*. Bruce *et al.* (1903a) described two cases of a disease ‘not unlike Trypanosoma fever’ in two immigrants who had recently come to Uganda (whom he termed Nubians). These were a policeman and a prisoner who had arrived from the present day Sudan (where *T. b. gambiense* occurs today). Bruce *et al.* (1903a) later insisted that the ‘Trypanosoma fever’ symptoms were simply the first stage of full blown sleeping sickness caused by *T. gambiense*.

5.1.3 Hypothesis

The analysis that follows tests the hypothesis proposed by Köerner *et al.* (1995), that the parasite responsible for sleeping sickness in the Busoga and surrounding regions
of Uganda from 1901 was due to *T.b. rhodesiense*. It is based on the examination of a set of clinical records from the time, and the comparison of the information obtained from these to recent epidemiological parameters collected in Tororo, southeast Uganda. In the present day, *T.b. gambiense* and *T.b. rhodesiense* are separated by a clear geographical boundary, that of the western Rift Valley (see Figure 1.2), and geographical location is a major factor contributing to the differential diagnosis of both diseases. The essential clinical differences between *T.b. gambiense* and *T.b. rhodesiense* sleeping sickness have been discussed in Chapter I; typically, *T.b. rhodesiense* is an acute disease, resulting in death after a period of 3-12 months (Apted, 1970; Odiit et al., 1997b). *T.b. gambiense*, on the other hand, is a chronic infection, with which an individual may continue to go about daily activities for many months or years (Apted, 1970; Taelman et al., 1987), despite suffering occasional and often mild symptoms.
5.2 Methods

5.2.1 Archives

The Mengo Hospital archives (C.M.S. Mission Hospital at Mengo) which encompass original patient case notes made by the Cook brothers, are held in the archives section of the Mulago Hospital/Makerere Medical School in Kampala, Uganda. The Mengo Hospital itself is located in a suburb of present day Kampala, and some of the original buildings remain. The first sleeping sickness case recognized in this facility was admitted on February 11th 1901.

The geographical distribution of the cases that presented to this hospital is not clearly defined, but can be surmised from the following “Bed Letter” referring to sleeping sickness cases and found amongst the archives. It is dated December 1909:

“The area from which the patients have been drawn is a very large one. Nassa at the south of the Lake has been mentioned, Nimule, 300 miles to the north (one case), Nairobi and Kikuyu 400 miles to the East furnished 3 patients, and many came from Busoga, Bunyoro, Toro and intervening parts. The large bulk of the patients, however, came from an area of which the centre is Mengo and the circumference a line drawn around with a radius of 100 miles.”

A number of cases that presented to Mengo would not have been recorded in these archives, especially in the latter years of the epidemic. This was mainly due to the sheer numbers of patients and the limited resources of the CMS mission. Many were referred to the specialist hospital run by the Royal Society Sleeping Sickness Commission in Entebbe, or later to the sleeping sickness isolation camps on islands in Lake Victoria. Some details of the treatments prescribed and numbers of cases seen in these camps are available (Hodges, 1902; Cook, 1940), and patient details for cases seen by the Sleeping Sickness Commission are also available (Bruce et al., 1903a). In either case, however, the details were not recorded by the Mengo team.

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12 Many thanks to Mrs Eunice Sendikadiwa, Makerere Medical School Librarian, for allowing me access to the archives.
An unsuccessful search was carried out for both the archives of the Royal Society Sleeping Sickness Commission hospital (including at the Royal Society in London) and the records of the sleeping sickness isolation camps set up on islands on Lake Victoria. Some of the details of patients seen by the Royal Society Commission can, however, be found in Bruce et al. (1903a). As early as 1902, J.H. Cook (Cook, 1902) stated that sleeping sickness patients were no longer admitted to the Mengo hospital as a matter of routine. He also provides, in the same paper, basic parasitological notes for a number of patients, but as these were not admitted, they do not appear in the database of inpatients. The Mengo archives provide details, therefore, of only a small sample of sleeping sickness patients at the time. They are none-the-less useful in helping to answer questions about disease origin and pathogenesis.

5.2.2 Data collection

5.2.2.1 Archives

Data were collected from two sources among the Mengo archives, namely the case notes for each patient and the hospital in-patient register. The case notes provided full details for each patient, as well as clinical notes taken throughout the course of the patients’ hospital stay. The in-patient register provided only summary details. For cases admitted prior to 1902, data were only available from this register as all the case notes prior to 1903 were destroyed in a fire at the end of 1902 (Cook, 1945).

For each patient considered at the time, by the Cook brothers, to have been infected with sleeping sickness (based either on clinical signs or parasitological diagnosis), full details as they appeared in the archives were entered to a database (Microsoft Excel®), which was used for later analyses. This electronic database is available for consultation from the author and will be made available to the archivist in Mengo. Full details were entered for patients presenting up to and including 1910; beyond this time, most patients were referred to the Royal Society hospital, to the sleeping sickness isolation camps, or turned away. In addition, the authorities were managing
to bring the epidemic under control, and the number of cases generally was diminishing.

The case notes and in-patient records were supplemented by additional ‘peripheral’ data sources such as personal letters that are also held in the archives. The details of one early case (case six in the database) were indecipherable from the records, but appeared in a peer-reviewed publication of the time (Cook, 1901).

### 5.2.2.2 T.b. rhodesiense comparison

Odiit et al. (1997b) published data on duration of symptoms and case fatality rates for 30 patients presenting with *T.b. rhodesiense* to LIRI sleeping sickness hospital in Tororo between 1988 and 1990. The Odiit et al. (1997b) data are shown in Table 5.1.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Time in months</th>
<th>Case no.</th>
<th>Time in months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>0.75</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>0.75</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>0.75</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>28</td>
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</tr>
<tr>
<td>14</td>
<td>2</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>30</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 5.1: Comparative data (Odiit et al., 1997b) of duration of illness to death of sleeping sickness patients in Tororo, 1988-1990.

### 5.2.2.3 T.b. gambiense comparison

Although a number of published case histories for *T.b. gambiense* patients are available (Robinson et al., 1980; Taelman et al., 1987; Petru et al., 1988; Benhamou
et al., 1989; Otte et al., 1995; Kirchhoff, 1998), there are no published accounts of time to death following infection with this parasite. Indeed, although Adams et al. (1986) conducted post-mortems on 16 fatal T.b. gambiense cases, they state that there are few observed fatal cases in the literature and that duration of illness prior to death is 'rarely established.' Although survival analysis (see below) can not be carried out on data where no deaths have occurred, it is possible to compare the overall time of illness until treatment of the patients described in case reports to those suffering from other diseases. The T.b. gambiense data are shown in Table 5.2.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Time infected (months)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>144</td>
<td>Kirchhoff (1998)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>24</td>
<td>Otte et al. (1995)</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>36</td>
<td>Robinson et al. (1980)</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>25</td>
<td>Petru et al. (1988)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>24</td>
<td>Taelman et al. (1987)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>72</td>
<td>Taelman et al. (1987)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>38</td>
<td>Taelman et al. (1987)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>13</td>
<td>Benhamou et al. (1989)</td>
</tr>
</tbody>
</table>

Table 5.2: T.b. gambiense cases with reported period of infection

5.2.3 Statistical analyses

5.2.3.1 Survival analysis

A survival analysis (Parmar & Machin, 1995) was conducted to compare the total time from infection to death of the patients in both 1901-1910 and in Tororo between 1988 and 1990. The criteria for inclusion of a patient from the archive dataset were that the patient had been recorded as a sleeping sickness death following in-patient stay in the hospital, that the length of the hospital stay was recorded and that the length of time of illness prior to admission had been recorded in the clinical notes. That is, an estimate of the total time of the clinical course of the illness, from onset of symptoms to death, was available.

Survival analysis is a technique designed to compare times from one point event to another in a group of subjects (such as time from infection with a pathogen to death or recovery), and is thus ideally suited to the analysis of the Mengo and Odiit et al.
(1997b) datasets available in this study (Parmar & Machin, 1995; Altman, 1997). It may also be useful for the analysis of right-censored datasets, that is, when a proportion of, say, the patients, do not die and therefore do not present a temporal endpoint in the analysis. Other forms of statistical analysis deal poorly with this sort of data. This strength of survival analysis techniques is not exploited in this study, as the end point, death, is known for all the patients. The variable lengths of time between onset of symptoms and death never-the-less make it a useful technique. For data such as the time of illness of T.b. gambiense patients, in which no deaths occurred, survival analysis is not a valid methodology. Essentially, the probability of a patient surviving to a given time following the self-reported onset of symptoms is calculated. The Kaplan-Meier survival estimate $S(t)$, or the probability of surviving until time $t$ (also called the product-limit estimator), is given by

$$S(t) = \prod_{t} \left(1 - \frac{d_{t}}{n_{t}}\right)$$

(Equation 1)

The expression $(1-d_{t}/n_{t})$ is evaluated at each occurrence of a death, and $S(t)$ is, in effect, the product of multiplying this function each time a death occurs. $d_{t}$ is the number of deaths between time intervals, and $n_{t}$ is the number of patients alive at the start of a time interval. The survival analysis was conducted in S+ 2000 (MathSoft, Inc.), and the survival curves were compared using the log-rank (Mantel-Cox) test.

### 5.2.3.2 Mann-Whitney-U test

The Mann-Whitney-U test, conducted in MINITAB version 13.1 (Minitab Inc.), was used to compare the length of illness until diagnosis of the eight T.b. gambiense cases to the length of illness until death of the T.b. rhodesiense and Mengo archive datasets. This test is suited for non-parametric data (Altman, 1997). These patients were all immigrants or visitors to Europe or the USA and in this study, the date of infection has been recorded as the date of the last visit to Africa. Cases of congenital T.b. gambiense sleeping sickness were excluded, and $p$-values less than .05 were considered significant.
5.3 Results

5.3.1 Descriptive statistics

Between 1901 and 1910, 11,767 patients were recorded in the Mengo in-patient records. This excludes all patients admitted in 1902 as all the records from that year were destroyed in a fire. Just over 1% of these (204 cases) were sleeping sickness cases. The age distribution of the sleeping sickness cases is shown in Figure 5.1. The male:female sex ratio was 3:1. The age-distribution of recent sleeping sickness cases in Soroti is shown in Figure 3.2. *T.b. gambiense* studies (Stanghellini & Duvallet, 1981; Pépin et al., 1994; Meda et al., 1995) have also shown a similar age-prevalence for that parasite, reflecting similarities in exposure risks to both parasites.

![Figure 5.1: Age distribution of 204 sleeping sickness cases in Mengo](image)

The outcome of admission was biased in favour of discharge (see Figure 5.2). Five patients were referred to the Entebbe sleeping sickness hospital, and 24 deaths recorded. Of these 24 deaths, 12 (details shown in Table 5.3) had complete records of date of admission and death, and of the amount of time ill prior to admission. Details for the eight *T.b. gambiense* cases are shown in Table 5.2.
Figure 5.2: Outcome of admission for 204 probable sleeping sickness patients reporting to the Mengo hospital between 1901-1910.

Table 5.3: Time sick pre-admission and total time to death for 12 patients admitted between 1901 and 1910
5.3.2 Survival curves

The Kaplan-Meier Survivorship curves resulting from the analysis of this data are shown in Figure 5.3. The analysis was run twice. Case number 1 in the Mengo dataset, which had a time until death considerably longer than the other patients, was included on the first occasion, and excluded on the second. The Tororo 1988-1990 dataset was kept constant in both analyses. The median survival times were 2.0 months, 3.8 months and 3.8 months for the Tororo 1988-1990, Mengo (with the 75 month case) and Mengo (without the 75 month case) datasets respectively.

5.3.3 Log-rank test

The log-rank test showed no significant difference between the Mengo and Tororo 1988-1990 survival rates, when computed with both the 75 month case ($\chi^2 = 1; d.f. = 1, p>0.05$) and without ($\chi^2 = 0.1; d.f. = 1, p>0.05$). Therefore, the clinical course from onset of symptoms to death in this sample of patients from the 1901-1910 epidemic in southern Uganda was not significantly different to that of patients reporting to LIRI sleeping sickness hospital in Tororo between 1988 and 1990.

5.3.4 Mann-Whitney-U Test

A significant difference was found between the length of illness until treatment of the eight T.b. gambiense cases and the length of illness until death of the T.b. rhodesiense cases ($W=274; p<0.001$) reported by Odiit et al. (1997b). A significant difference was also found between the eight T.b. gambiense cases and the Mengo archive cases, both with ($W=122; p<0.05$) and without the 72 month case ($W=121; p<0.001$).
Figure 5.3: Kaplan-Meier survival curves. Green lines denote $S(t)$, dotted lines are 95% confidence intervals, calculated by the log-rank method. a) Published data from Odilt et al. (1997b); Mengo archives relating to the period 1901-1910 including b) 75 month and c) excluding 75 month case.
5.4 Discussion

The clinical picture of sleeping sickness, measured in terms of the time to death from infection, does not differ between the *T.b. rhodesiense* cases that presented in the last decade in Tororo (Odiit et al., 1997b) and sleeping sickness cases that presented at the Mengo Mission Hospital during the period 1901-1910. In addition, the duration of illness of documented *T.b. gambiense* cases did differ significantly from both the Mengo patients and Tororo patients. From this, it can be concluded that the patients included in the analysis from the 1901-1910 epidemic were probably infected with trypanosomes belonging to the *T.b. rhodesiense* sub-species, and that this parasite was circulating in the region at the time. The hypothesis proposed by Köerner et al. (1995) appears to be correct.

Given these results, how might the observations made by Castellani (1903), that two distinct clinical pictures were seen, be explained? The designation as ‘Nubians’ of some of the cases he describes (see Section 5.1.2.1) suggests that these patients were from Sudan. They were migrants, and may well have been carrying *T.b. gambiense* parasites with which they were infected before migrating, and which were only discovered on examination in Uganda. Although local transmission of these parasites can not be excluded, *T.b. gambiense* is unlikely to have been responsible for the widespread deaths in the Busoga region generally. Most of the cases described by Christy (1903) as he roamed around Uganda were of an acute disease. Hodges, who was the Medical Officer for the Uganda Protectorate, states, based on a great many observations at the time, that the time to death of Ugandan patients from first realising they were sick was between 3-4 months (Hodges, 1902), and that the total duration of illness rarely exceeded 10 months. *T.b. gambiense*, if it did exist concurrently, would have been limited in distribution to those areas where migrant workers, working for the British authorities, were allowed to settle. These settlement areas were purposefully designated to be away from tsetse infested bush, in the efforts to control the epidemic (Anon., 1909).
Two points for discussion arise from the data themselves. The first, of historical interest, is that the sex ratio observed in the sleeping sickness patients during 1901-1910 favours males, possibly indicating greater priority given to males in seeking health care at the time. Secondly and more importantly, if both species of human infective trypanosome (T.b. gambiense and T.b. rhodesiense) were present in Uganda during the 1901-1910 epidemic, and both were presenting at the Mengo hospital, the data for deaths acquired from the archives would be biased towards clinical descriptions of T.b. rhodesiense. The comparative data for length of illness until treatment for T.b. gambiense show that most T.b. gambiense cases are treated before death occurs (the length of illness to death has proved difficult to come by for this disease as patients with T.b. gambiense die only rarely in hospital, when they present with very advanced stages of the disease). For similar reasons, T.b. gambiense would be poorly represented among deaths in Mengo – patients would have been discharged as the symptoms would not have been considered serious enough to allow them to remain in the scarce hospital beds. The case of sleeping sickness that was included in the analysis that was 72 months ill was possibly such a case that presented with T.b. gambiense in its final stages. The results of this analysis cannot therefore exclude T.b. gambiense as a cause of illness among some patients, but can positively include T.b. rhodesiense as a cause of sleeping sickness at the time. It is likely that the epidemic was a principally T.b. rhodesiense epidemic with occasional cases of T.b. gambiense in patients that had migrated from T.b. gambiense foci on the present north-western border of Uganda (for example, ‘Nubians’).

5.4.1 Rinderpest and cattle restocking

If, as these data suggest, the 1900-1910 epidemic was in fact due to T.b. rhodesiense, the question arises as to its causes, and of the cause of the spread of sleeping sickness through the whole of the region, to previously unaffected areas. There is no doubt that the onset of the colonial administration in Uganda resulted in social changes and population mobility (Ford, 1971; Musere, 1990), which also had important environmental consequences (Ford, 1971). Musere (1990), in particular, is damning of the colonial powers and the changes they made to the biogeography of southern Uganda. Lyons (1992), in referring to the causation of T.b. gambiense epidemics in
central Africa, also blames the disruptive effects of colonization. Human movements had, however, occurred regularly throughout Africa's history (Köerner, 1994; Köerner et al., 1995). Some other trigger, in conjunction with these factors, is likely to have been involved in spreading sleeping sickness from the endemic foci outwards. This might have been an infectious disease of livestock, rinderpest. The timing of the start of the sleeping sickness epidemic coincides with the end of the great rinderpest pandemic in cattle (Ford, 1971; Rossiter, 1994; Reader, 1997).

In the early 1890s, and for the decade that followed, rinderpest, or cattle plague, ravaged most of Africa, and eastern Africa was not spared (Reader, 1997). Millions of cattle died from this virulent viral infection (Rossiter, 1994), causing sociological and ecological upheavals throughout the continent. Rinderpest is rightly often linked to the sleeping sickness outbreaks in eastern Africa, although possibly for the wrong reasons. The disease-induced cattle depopulation is thought to have resulted in a change in the dominant vegetation in the whole region; pasture lands reverted to bush and the distribution of tsetse expanded (Waller, 1990; Reader, 1997). Although these ecological changes certainly occurred, they may not have been directly responsible for the spread of sleeping sickness.

In Chapters II and III, it has been shown that the movement of cattle during livestock restocking may be linked to the introduction of sleeping sickness to previously unaffected areas, resulting in serious outbreaks of disease. With the large scale movements of animals that would have occurred after the rinderpest pandemic, as animals were moved across ecological zones and traded in the cattle-depopulated areas, trypanosomes would have been moved with them. In conjunction with the expansion of tsetse distributions as the ecology changed, the setting was ripe for a major sleeping sickness problem. Ford (1971) notes that in the setting up of the Uganda Protectorate, there was a great deal of cattle movements, either for groups moving away from areas under British control or in search of post-rinderpest pasture. He also points out that Rhodesia, now Zambia, was restocked after rinderpest, by trade routes from north of the Zambezi river, and discusses the fact that cattle routes and the culture of large scale cattle trading over great distances was well established.
These facts are indicative of great regional cattle movements. *T.b. rhodesiense* could have spread from Busoga and other endemic foci in Buganda all around the northern Lake Victoria shore with the post-rinderpest restocking.

Conventionally, *T.b. rhodesiense* is thought to have spread through east Africa and to Uganda from Zambia (MacKichan, 1944). However, the data presented in Chapter IV and elsewhere (Hide *et al.*, 1996; Hide *et al.*, 1998) suggest that the parasite retains a stable genetic constitution through time. Strains from Zambia have been shown (Hide *et al.*, 1991; Hide *et al.*, 1994) to be quite distinct from *T.b. rhodesiense* in Uganda. It is therefore unlikely that the parasite spread from there to Uganda. Rather, as Köerner *et al.* (1995) suggested, *T.b. rhodesiense* has probably been present, either at endemic or epidemic levels, in southeast Uganda for many hundreds of years. The results obtained in Chapters II, III and IV explain the spread of sleeping sickness in present day East Africa; the dynamics of the spread of the disease have probably been similar since the first association of cattle and humans in tsetse infested areas of this part of the continent.
Chapter VI:
General discussion
This thesis has explored the epidemiology of sleeping sickness caused by *T.b. rhodesiense* in Uganda, and has focussed on the interactions between the parasite reservoir and humans to explain the spread of the disease. Chapters II-IV examined the origins of a recent sleeping sickness outbreak in Soroti District, assessing the risk of introducing parasites to parasite-free areas (as well as assessing methods of preventing this in the future), observing the geographical spread of the disease from a point source and suggesting the underlying causes of this spread, and finally explaining the phylogenetic origins of the parasites involved and examining trypanosome population structures in the region using molecular epidemiological techniques. In Chapter V, evidence was presented to support the hypothesis that the 1900-1910 epidemic of sleeping sickness in Uganda was, in part at least, due to *T.b. rhodesiense*, which may have spread as a result of the same processes that resulted in the establishment of sleeping sickness more recently in Soroti.

### 6.1 Disease spread

*T.b. rhodesiense* parasites have clearly spread beyond the established endemic foci in southeast Uganda as a result of the movement of livestock, and have become established in Soroti. Cattle movements originating in both sleeping sickness-affected and sleeping sickness-free areas are continuing on a wide scale across Uganda, well beyond the boundaries of the outbreak described, as part of the national restocking programme. Sleeping sickness is therefore an actively re-emerging zoonosis in the region, particularly where livestock, humans and tsetse interact. Early results from follow-up surveys connected with this thesis (E.M. Fève, pers. obs.) have demonstrated that in a village well outside the Serere focus (approximately 40 kilometres north of the outbreak described in Chapter III), cattle are carrying *SRA* positive infections (K. Picozzi, pers. com.). It is likely that these infections have also been imported with cattle as this area, in parallel to the situation in Serere before 1998, has never reported sleeping sickness. The results of the analysis in Chapter II show that, as cattle movements continue through time in other parts of Uganda, there is a real possibility of importing sufficient human infective parasites to start further outbreaks. This risk exists anywhere within the tsetse belt
where cattle movements are ongoing. Control programmes must therefore account for the potential spread of sleeping sickness, and put in place appropriate interventions.

6.1.1 Demographic factors: humans and livestock

The analysis of the cattle trade into Soroti and the spatial distribution of sleeping sickness around Brookes Corner livestock market, is consistent with the hypothesis that cattle carried human infective trypanosomes into Soroti District; in contrast, there was no evidence showing that infected humans imported the parasite. In general terms, population movements, both of livestock and of humans, are significant risk factors for the spread of directly transmitted and vector-borne diseases. Human population movements have been identified as significant in the epidemiology of malaria due to both *P. falciparum* and *P. vivax* in the Mekong delta region of Southeast Asia (Kidson *et al.*, 1999), and have been implicated in the spread of *T.b. gambiense* for which the principal reservoir is humans (Jannin *et al.*, 2001; Sonne, 2001; Moore *et al.*, 2002), although the magnitude of the impact of these movements on *T.b. gambiense* epidemiology remains unquantified. Animal population movements, both domestic and wild, are no less important in the spread of many other diseases affecting them. For example, *Trypanosoma evansi* may have spread through the Philippines with movements of buffalo in livestock breeding programmes (Reid, 2002); elimination of rabies in the European fox population has encountered setbacks due to the mobility of foxes across national borders (Breitenmoser *et al.*, 2000); foot-and-mouth disease is thought to have spread rapidly in the UK during 2001 partly as a result of the large-scale movements of domestic livestock (Woolhouse *et al.*, 2001a); the introduction of beef animals to dairy herds is thought to be a factor in the genesis of outbreaks of bovine tuberculosis caused by *Myobacterium bovis* (Marangon *et al.*, 1998). Understanding the demography of human and livestock populations and other reservoirs of re-emerging and zoonotic diseases is fundamental to understanding the epidemiology of these diseases.
6.2 Disease prevention

Vector control in the form of fly trapping was undertaken at the very start of the outbreak in Serere, and while such activities remain an important option for sleeping sickness control in the area, they may not be cost effective to maintain in the resource-poor rural communities affected by sleeping sickness. In particular, the cost of extending vector control over a larger area may become prohibitive and unsustainable. Sleeping sickness in Serere may now have spread far enough from Brookes Corner market to make this the case even there. In Uganda, the cost of one impregnated tsetse trap is approximately US$ 15 using imported materials and US$ 7 using local materials (B. Dransfield, pers. com.), and ten traps are required for each square kilometre to be effective at reducing fly densities sufficiently to interrupt sleeping sickness transmission during an epidemic (Lancien, 1991). Okoth et al. (1991) have demonstrated some limited success with community-based tsetse trapping interventions in Tororo using local materials, but these are unlikely to ever achieve the extensive coverage required. Other wide-area methods for tsetse control, such as the release of sterile male flies, may be successful in confined areas (IAEA, 1997), but are unlikely to present a cost-effective and sustainable solution for long-term disease management (Molyneux, 2001a), in terms of both livestock and human trypanosomiasis. Pour-on insecticides, targeted at tsetse but applied to cattle, are another potential control option in some settings. This method of control is expensive, however (Kamau et al., 2000), and may not always be effective at reducing trypanosome prevalence over large areas (Rowlands et al., 2001). In particular, the effective long-term use of pour-ons has been shown to require coverage of an unsustainably high proportion of the cattle population (Okiria et al., 2002).

6.2.1 Treatment of cattle

The treatment of cattle is an important option for preventing the importation to, and transmission of human infective parasites in unaffected areas. Control strategies such as the obligatory testing and treatment of cattle with a single curative dose of a trypanocide prior to sale in markets have been evaluated in terms of risk reduction in
Chapter II, and more generally by Welburn *et al.* (2001a). A major trade-off to consider is the potential adverse effect such a strategy would have on the selection of drug resistance on the one hand (see Section 6.2.2, below) and the benefits of animal treatments as a public health prevention measure, in terms of DALYs averted, on the other. The DALY impact of *T. b. rhodesiense* sleeping sickness was highlighted in Chapter I, and any intervention that might substantially reduce the disease burden should be given consideration. Treating cattle would have the added effect of improving animal health by clearing other, non human-infective trypanosomes of livestock such as *Trypanosoma vivax* and *T. congoense*. A full cost-effectiveness analysis of the possible interventions is required and must thus consider both medical and veterinary inputs and outcomes. Cost-effective prevention strategies are a necessity in countries such as Uganda where human and animal health services are increasingly decentralised and where few funds are available generally for health improvement activities (Jeppsson & Okuonzi, 2000; Jeppsson, 2001; Bossert & Beauvais, 2002). Molyneux (2001b) highlights the conflict between the trend towards decentralisation and the need for a vertical approach to sleeping sickness control.

Interventions aimed at domestic livestock which ultimately improve human health are also relevant in other contexts. Hewitt and Rowland (1999) and Rowland *et al.* (2001) treated cattle with deltamethrin pour-on insecticides to prevent transmission of malaria by zoophilic mosquitoes in peridomestic habitats in Pakistan, and the effective control of schistosomiasis caused by *Schistosoma japonicum* may depend, in some areas, on control of the parasite in the cattle reservoir (Guo *et al*., 2001). Understanding the biology and epidemiology of zoonotic diseases in their reservoir hosts (Cleaveland *et al*., 2001; Woolhouse *et al*., 2001b) is essential to the appropriate design of public health interventions and disease control programmes.

The implementation of integrated control, targeting cattle, humans and possibly tsetse, would benefit from co-ordination between veterinary and medical authorities, and such intersectorial collaborations, leading to improved human and animal health, would contribute generally to rural development in tsetse infested countries across
East Africa. Some of the findings of this thesis (Fèvre et al., 2001) have already led to a potential implementation of a control policy – that cattle from sleeping sickness risk areas should be treated prior to migration as part of restocking activities (Wendo, 2002). Although it remains unclear what treatment regimens might be used, the initial suggestion is for the farmers or traders involved in restocking to pay for these treatments themselves.

6.2.2 Drug resistance

The development of drug resistance threatens the effective control of many diseases, such as tuberculosis, HIV/AIDS and malaria. Mass treatment, advocated as a useful trypanosomiasis control tool in Chapter II, is one of the many ways by which drug resistance may be selected. For example, mass treatment of *P. falciparum* malaria through chloroquinized salt in Cambodia in the early 1960s (Verdrager, 1986) may have provided the appropriate conditions for resistance to chloroquine to develop. Geerts *et al.* (2001) argue that preventing drug resistance in animal trypanosomes may in fact depend on limiting treatments to clinical cases, as mass treatment of herds may impose a strong selection pressure on the trypanosome population (Matovu *et al.*, 2001b). The potential impacts of mass drug use on trypanosome drug resistance in Uganda need therefore to be considered in the formulation of control policies. Drug resistance mechanisms are poorly understood for the trypanosomes (Barrett, 2001); there is evidence in laboratory strains, however, to suggest that there may be cross-resistance between diminazene (drug of choice for therapeutic – rather than prophylactic - treatment of cattle) and melarsoprol (most significant drug for late stage sleeping sickness, especially due to *T.b. rhodesiense*), as the mode of uptake of both compounds by trypanosomes is similar (Barrett & Fairlamb, 1999). In both cases, uptake of the drugs is through an amino-purine transporter across the trypanosome membrane (termed the P2 transporter), first described by Carter and Fairlamb (1993).

If anti-trypanocidal drugs are used appropriately and in a controlled manner during mass drug administration, the impact on the development of drug resistance would be limited while the reduction in the risk of spreading human infective parasites to
previously non-sleeping sickness areas within the Ugandan tsetse belt would be substantial. This requires, as already mentioned, collaboration between medical and veterinary sectors in the formulation of policy. A field diagnostic test based on the presence of the SRA gene or the protein product for which it codes (Welburn et al., 2001b), would be of great value in this context, as animals carrying T.b. rhodesiense could be targeted selectively in livestock markets, further reducing the selection pressure that could result from the use of mass treatments.

6.3 The way ahead

Research on disease control and prevention must continue in those regions of Uganda that lie within the tsetse belt and that are currently, or scheduled to be, involved in restocking activities. Monitoring of cattle movements, and an in-depth understanding of the economic and social factors that drive the cattle trade from one region to another are necessary, both during and outwith periods of restocking. In addition, the risk of future outbreaks must be assessed in conjunction with landuse issues, by building a geographical information system in which economic variables, human and cattle demography and landuse come together. Sentinel sites, such as key cattle markets, might be identified for long term monitoring of infections in the highly mobile cattle population. Satellite imagery analysed in a geographical information system is an important epidemiological tool (Beck et al., 1994; Pope et al., 1994; Thomson et al., 1999; Beck et al., 2000; Zhou et al., 2001), in particular in relation to landuse and vector-borne disease risk. Assessing trypanosomiasis risk by seeking associations between disease prevalence and remotely sensed variables has already been carried out at a continental scale (Rogers & Williams, 1993; Rogers, 2000), and on a regional scale (Robinson et al., 1997; Robinson, 1998; Hendrickx, 1999; Hendrickx et al., 2001), mainly using entomological parameters. Local scale work using remotely sensed satellite imagery and detailed geo-referenced databases has been successful in Burkina Faso, in assessing the risk from animal trypanosomiases (de la Rocque, 1997; de la Rocque et al., 1999; Michel et al., 1999). Combining the technologies used for large-scale and local-scale studies would provide new insights into the epidemiology of both human and animal
trypanosomiasis in Uganda and other East African trypanosomiasis foci. In particular, predicting the spread of the parasite and the local expansion of foci in newly affected areas would be of value to public health initiatives.

6.3.1 The immediate future

Areas of Uganda of particular concern with regard to the future spread of *T. b. rhodesiense* sleeping sickness include the districts of Lira, Apac and Masindi on the northern side of Lake Kyoga (see Figure 1.4). Masindi may already be affected; Enyaru *et al.* (1999) passaged one trypanosome isolate derived from a human in Masindi through mice, and on the basis of the short pre-patent period and novel isoenzyme profile concluded that the parasite was *T. b. rhodesiense*. The identity of this parasite isolate has not yet been confirmed using the SRA methodology (Welburn *et al.*, 2001b). It remains, however, that the Lake Kyoga basin, which is well within the *G. f. fuscipes* fly belt (see Figure 3.6), and where extensive cattle movements are occurring, presents an area for possible spread of *T. b. rhodesiense*. Masindi and Lira are of particular interest as they border the *T. b. gambiense* focus in north western Uganda, and the spread of *T. b. rhodesiense* to this area would result in mixing of *T. b. rhodesiense* and *T. b. gambiense* in the same human population and transmitted by the same fly population. This could have serious implications, not least because of the different control policies required for the different diseases. Other than the possible co-habitation of the sub-species during the 1901-1910 epidemic (see Chapter V), this would be the first instance where the two sub-species were sharing an ecological niche.
References


Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (SRA) gene

S C Welburn, K Picozzi, E M Fèvre, P G Coleman, M Odii, M Carrington, I Maudlin

Summary
Background The expansion of sleeping sickness caused by Trypanosoma brucei rhodesiense beyond its traditional focus in southeast Uganda has been linked with large-scale livestock restocking. To assess the risk presented to the human population by domestic livestock, human-infective T b rhodesiense must be distinguished from non-human-infective T brucei brucei, since both parasites can be present in cattle. We investigated the use of a simple genetic marker to characterise parasites collected from cattle in villages within the new sleeping sickness focus in Soroti District, Uganda.

Methods 70 T brucei si samples of known human infectivity status collected from human beings and cattle in Tororo District, Uganda, from 1989 to 1991 were screened for the presence of the human-serum-resistance-associated (SRA) gene by conventional PCR. In 2000–01, blood samples from 200 randomly selected cattle in six villages and two markets in Soroti District were screened for T brucei si parasites by PCR; positive samples were screened for the presence of the SRA gene.

Findings The SRA gene was present in all 29 samples from patients with sleeping sickness in Tororo District. Of the 41 samples collected from cattle at the same time, the SRA gene was present in the eight samples that tested resistant to human serum in vitro, whereas it was absent from all 33 isolates that were sensitive to human serum in vitro. Of the 200 cattle sampled in Soroti District, we estimated that up to 18% (95% CI 12–23) were infected with T b rhodesiense.

Interpretation Detection of the SRA gene could provide the basis for a simple diagnostic test to enable targeted control of T b rhodesiense in the domestic livestock reservoir, thereby reducing the public-health burden of sleeping sickness in east Africa.

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Introduction
Sleeping sickness is endemic in east Africa and has, in the past, given rise to epidemics on a large scale. At the turn of the past century, more than 500 000 people are estimated to have died during an epidemic in southeast Uganda.1 Sleeping sickness, caused by Trypanosoma brucei rhodesiense and transmitted by the tsetse fly, is fatal if not treated. Drug treatment can be associated with severe side-effects, and up to 5% of people die as a result of chemotherapy.2 The disease continues to incur a significant public-health burden in sub-Saharan Africa, and a more in-depth understanding of its epidemiology is necessary for the development of appropriate control methods.

In southeast Uganda, domestic livestock are known to be a reservoir of human infective T b rhodesiense.3 The ability of cattle to maintain human infective trypanosomes for long periods poses a serious threat to the health of rural people at a time when increased use of cattle is desperately needed to intensify rural productivity.

During the past 2 years, there have been serious concerns about the spread of sleeping sickness to areas previously free of the disease—ie, north of the traditional disease focus in southeast Uganda. In December, 1998, a case of human sleeping sickness was detected in Soroti District, Teso region, for the first time.4 Since then, the disease has spread 25 km from the original focus, and caused more than 150 recorded cases (which probably represents only a small proportion of those truly affected, many of whom almost certainly did not present for treatment). A study in which hospital records were used to map the spread of the disease indicated that early cases were centred on the main cattle market, suggesting that restocking of cattle was responsible for the spread of sleeping sickness.4 The restocking of livestock in Teso has been identified as a central activity for the development of the region.

Calculation of the extent of T b rhodesiense carriage in cattle is difficult because the parasite that causes human disease is morphologically, serologically, and biochemically identical to another parasite, T brucei brucei, which is symptomless in animals but which cannot infect humans. Survival in human serum forms the basis of distinguishing human infective T b rhodesiense from non-infective T b brucei. Laboratory tests to distinguish the two are based on analysis of DNA (restriction-fragment-length polymorphism [RFLP] analysis) and in-vitro assays of serum sensitivity (human serum resistance [HSR] test)5,6. These methods are laborious, time consuming, and impractical for application to the field situation. The molecular differences between human infective T b rhodesiense and non-infective T b brucei are still not clearly understood, but ectopic expression of the
human-serum-resistance-associated (SRA) gene has been found to allow T. brucei to survive exposure to normal human serum. The discovery of a gene that confers resistance to human serum offers the prospect of a simple one-step diagnostic test for T. rhodesiense.

We aimed to validate the SRA gene as a diagnostic indicator of T. rhodesiense, and to apply the diagnostic test to calculate the prevalence of T. rhodesiense in the cattle population at the centre of the recent sleeping sickness outbreak in Soroti District.

Methods
70 T. brucei sl isolates were collected from sleeping sickness patients and cattle in an endemic focus of T. rhodesiense sleeping sickness in Tororo District, southeast Uganda, between 1988 and 1991 as previously fully described. Genomic DNA was prepared from all 70 isolates (29 human and 41 cattle). An in-vitro HSR test was done on all cattle isolates, and RFLP analysis was done on all cattle isolates and 16 of the human isolates. Cluster analysis assigned the RFLP-analysed samples to ten groups: groups 1–9 comprised T. brucei, and group 10 T. rhodesiense.

During 2000–01, we sampled 200 randomly selected cattle from six villages and two markets within a 25 km radius of Brooks Corner market—the epicentre of the sleeping sickness outbreak in Soroti District.

For the Tororo District isolates, 1 mL samples of trypanosomes that had previously been cryopreserved were thawed and centrifuged at 6000 rpm in a microfuge for 5 min. The pellet was then resuspended in 200 μL phosphate-buffered saline. Genomic DNA was isolated by use of the QIAamp DNA Blood MiniKit (Qiagen, Crawley, UK); DNA was eluted in TE buffer.

For the Soroti District samples, DNA was prepared directly from cattle blood. Genomic DNA was isolated from heparinised blood by use of DNAzol BD Reagent (Invirogen, Groningen, Netherlands) and resuspended in TE buffer.

Primer used to amplify species-specific DNA targets for T. brucei sl were as follows: TBR (30 cycles) 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; Orphon (40 cycles) 94°C for 30 s and 68°C for 90 s; Musq (30 cycles) 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s. PCR products were visualised by electrophoresis through 1.5% (w/v) agarose containing 0.5 mg/L etidium bromide.

All isolates from Tororo and Soroti districts were first confirmed as T. brucei sl by established PCR techniques, and were screened for the presence of the SRA gene. The specificity of the SRA screening assay was dependent on the design of the primers and on the length of the amplification product. The T. brucei genome contains variable surface glycoprotein genes that have some sequence identity with SRA. Compared with these variable surface glycoprotein genes, SRA has an internal deletion of 378 bp that encodes the central part of the variable surface glycoprotein N-terminal domain. The PCR primers were designed to amplify across this deletion and thus distinguish the SRA product (743 bp) from any SRA-related variable surface glycoprotein (1121 bp).

Amplification of the SRA gene was done with primers B573 (CCATGGCCCTTGGACGAAGACCCGC) and B578 (CTCGAGTTTTTCGTTATTTCCTCC) at 2 μM/L. The primers were based on the published SRA gene sequence (EMBL accession number AF097331). The amplification programme was dependent on the source material: DNA purified from Tororo samples, enriched for parasite DNA by mouse passage, was amplified through 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s; DNA isolated directly from cattle blood from Soroti contained predominantly bovine DNA and was amplified through 10 cycles 94°C for 20 s, 55°C for 30 s, and 72°C for 60 s, followed by 25 cycles of 94°C for 20 s, 63-8°C for 30 s, and 72°C for 60 s (the time at 72°C was increased by 2 s per cycle). Routinely, 25 μL reaction volumes were used, which contained either 1:25 U TaqBead Hot Start polymerase (Promega, Southampton, UK) or a combination of 1 U HotStarTaq DNA polymerase (Qiagen) supplemented with 1 U Pfu polymerase (Promega) and 4 mmol/L MgCl2. In each individual experiment, positive controls for T. brucei, T. rhodesiense, and T. gambiense were used. The identity of the PCR product was confirmed by cloning directly into the plasmid pGEM-T Easy (Promega), and sequencing at the Molecular Genetics Analysis Facility, Ninewells Hospital, Dundee, UK. All SRA PCR products sequenced contained intact open reading frames and had more than 99% identity with the Genbank SRA sequence.

Results
The results of the various characterisation analyses done on the 70 isolates from Tororo District are shown in the table. The SRA gene was present in all isolates that were positive in the HSR tests and absent from all the samples that were negative in HSR tests. All 29 isolates from human beings with sleeping sickness were positive for the SRA gene. Additionally, eight isolates from cattle that were shown to be resistant to human serum in vitro were also positive for SRA. The SRA gene was absent from all 33 cattle isolates that were sensitive to human serum in vitro. Of the 200 cattle sampled in Soroti District, 89 (43%) tested positive for the presence of T. brucei sl by PCR. Of these, the DNA from 45 cattle were sufficiently well preserved to be screened for the SRA gene, and 18 of these 45 (40%) were SRA positive and therefore characterised as T. rhodesiense. The minimum prevalence of SRA was 9% (18 of 200; 95% CI 5–13). However, assuming that the SRA gene was present in the same proportion of those 44 samples from cattle infected with T. brucei sl but not screened for the SRA gene, the prevalence of T. rhodesiense in cattle was estimated to be about 18% (95% CI 12–23).

<table>
<thead>
<tr>
<th>Test</th>
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<th>Cattle</th>
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<tbody>
<tr>
<td>HSR test</td>
<td>Positive</td>
<td>29</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>RFLP analysis</td>
<td>Group 10</td>
<td>10†</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Groups 1–9</td>
<td>0</td>
<td>31</td>
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<tr>
<td>PCR detection of SRA</td>
<td>Positive</td>
<td>29</td>
<td>8*</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
<td>33</td>
</tr>
</tbody>
</table>

Human serum resistance, RFLP = restriction-fragment-length polymorphism.

*The same eight cattle isolates were human-serum-resistant in vitro and SRA positive. Only 16 of the 29 human isolates were RFLP analysable.†The two cattle isolates assigned by RFLP to group 10 were human-serum sensitive in vitro and SRA negative.

Comparison of screening for the SRA gene by PCR analysis with characterisation by isolation from a human being, the HSR in-vitro test, and RFLP analysis of 70 T. brucei sl isolates from Tororo District, Uganda
Discussion

Aside from isolation directly from human beings, trypanosome sensitivity to human serum is regarded as the benchmark for human infectivity. We have shown that in vitro HSR and SRA test results for the previously characterized Tororo District samples were identical. The presence of the SRA gene seems to be sufficient within the southeast Uganda sleeping sickness focus to discriminate between T b rhodesiense and T b brucei.

If expression of SRA is necessary to confer resistance to human serum, detection of the SRA gene in cattle isolates that were phenotypically resistant to human serum in vitro is suggestive of continued gene expression even outside the human host. The results described here contain a minor inconsistency with the findings of Hide and colleagues. Previously, two isolates from cattle (Tira 68 and Tira 17) had been grouped with the human-serum-resistant genotypes by RFLP analysis despite being sensitive to human serum in vitro. The finding here that these two isolates do not contain the SRA gene is consistent with the serum sensitivity test and also with minisatellite data analysis that suggested these same two stocks were not human-serum resistant, and that they are T b brucei.

Application of the SRA test to recent field samples from an active sleeping sickness focus suggested that up to 18% of domestic cattle within the focus are now infected with human infective T b rhodesiense. These results are consistent with the finding that cattle are the major reservoir of human sleeping sickness in eastern Uganda. Moreover, the results underline findings that movement of infected animals is more important than migration of infected human beings in the spread of sleeping sickness throughout time-endemic areas. Uganda is the only country to have endemic foci of both infectious agents of sleeping sickness, T b rhodesiense and T b gambiense. The recent re-emergence of both forms of sleeping sickness across sub-Saharan Africa and the geographical spread of T b rhodesiense suggest that there could be a significant overlap between T b gambiense, which is believed to be primarily anthropoendemic, and T b rhodesiense in Uganda. In the event of such overlap, testing for the SRA gene, which is absent in T b gambiense, will be invaluable in ensuring that inappropriate treatments are not prescribed (the drugs for late-stage sleeping sickness differ for the two forms of the disease). In light of the findings presented here, we are in the process of sampling domestic livestock across Uganda and other sleeping-sickness foci across east Africa to determine the spatial distribution of T b rhodesiense.

The central role of cattle in the spread and persistence of T b rhodesiense sleeping sickness provides important opportunities for human disease control by targeting the animal reservoir, for example through chemotherapy. Affordable and effective veterinary trypanocides are readily available and widely used throughout Africa. However, the emergence of drug resistance is an increasing problem. It, as recently suggested, the mechanism for resistance to the major veterinary drug diminazene aceturate is similar to that involved in resistance against the human drug malarospor, widespread veterinary drug use could affect animal health and public health. The use of SRA detection as a diagnostic tool provides the opportunity to target drug use to cattle infected with T b rhodesiense, thereby reducing the transmission of the parasite to human beings while keeping the potential for drug resistance to a minimum. We suggest that efforts should be directed at the development of appropriate diagnostic and control strategies that take into account the risk presented by the animal reservoir and the opportunity that SRA screening presents.

Contributors

The study was conceived by S C Welburn, P G Coleman, and I Maudlin. The Tororo District samples were collected by I Maudlin, S C Welburn, and M Odi. The Soroti District survey was developed by S C Welburn, P G Coleman, and E M Fèvre, and carried out by E M Fèvre and K Picouzet. Molecular analysis was done by S C Welburn, M Carrington, K Picouzet, and E M Fèvre. All investigators were responsible for manuscript preparation.

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References

The origins of a new Trypanosoma brucei rhodesiense sleeping sickness outbreak in eastern Uganda

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Summary

Background Sleeping sickness, caused by two trypanosome subspecies, Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense, is a parasitic disease transmitted by the tsetse fly in sub-Saharan Africa. We report on a recent outbreak of T b rhodesiense sleeping sickness outside the established south-east Ugandan focus, in Soroti District where the disease had previously been absent. Soroti District has been the subject of large-scale livestock restocking activities and, because domestic cattle are important reservoirs of T b rhodesiense, we investigated the role of cattle in the origins of the outbreak.

Methods We identified the origins of cattle entering the outbreak area in the 4 years preceding the outbreak. A matched case-control study was conducted to assess whether the distance of villages from the main market involved with restocking was a risk factor for sleeping sickness. We investigated the spatial clustering of sleeping sickness cases at the start of the outbreak.

Findings Over 50% (1510 of 2796) of cattle traded at the market were reported to have originated from endemic sleeping sickness areas. The case-control study revealed that distance to the cattle market was a highly significant risk factor for sleeping sickness (p<0.001) and that there was a significant clustering of cases (27 of 28) close to the market at the start of the outbreak (p<0.001). As the outbreak progressed, the average distance of cases moved away from the cattle market (0.014 km per day, 95% CI 0.008–0.020 km per day, p<0.001).

Interpretations The results are consistent with the disease being introduced by cattle infected with T b rhodesiense imported to the market from the endemic sleeping sickness focus. The subsequent spread of the disease away from the market suggests that sleeping sickness is becoming established in this new focus. Public health measures directed at controlling the infection in the animal reservoir should be considered to prevent the spread of sleeping sickness.

Lancet 2001; 358: 625–28
See Commentary page 603

Introduction

Sleeping sickness is a parasitic disease, unique to sub-Saharan Africa, imposing a considerable public health burden on affected rural populations.1 Two protozoan subspecies, Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense, both transmitted by the tsetse fly (Glossina spp), cause the disease, which is fatal if untreated.2,3 Uganda is the country of origin of both parasites, T b gambiense in the north-west and T b rhodesiense in the south-east of the country.4 A new T b rhodesiense sleeping sickness outbreak has been identified in an area outside the established focus.

The spatial distribution, pathogenesis, and epidemiology of sleeping sickness caused by the trypanosome subspecies differ notably.4 T b gambiense tends to occur in western and central Africa, causing a chronic infection that can last several years. T b rhodesiense infections, by contrast, are restricted to eastern Africa and are characteristically acute, with untreated cases dying within 3–6 months of infection.5 An animal reservoir is considered essential to the long-term persistence of T b rhodesiense sleeping sickness,6,7 while the importance of a non-human reservoir in the epidemiology of T b gambiense is less certain.8 Evidence from studies conducted in south-east Uganda implicates domestic livestock, particularly cattle, as the principal reservoir of T b rhodesiense parasites.9

Data from previous studies in south-east Uganda, where Glossina fuscipes fuscipes is the vector, reported the prevalence of T brucei species in the domestic cattle population to be 5%, and of these, 23% were human infective. T b rhodesiense is the more human infective.9 Neither T b brucei nor T b rhodesiense are pathogenic to cattle. These data also showed that a tsetse fly was five times more likely to pick up an infection from a cow than from a human.

There have been three major epidemics of sleeping sickness recorded in south-east Uganda since the 1890s.10 The documented geographic extent of T b rhodesiense transmission in this region has, however, remained fairly unchanged for the past century, with cases confined to the area extending east of Kampala up to the Kenyan border, north of Lake Victoria and south of Lake Kyoga (figure 1). In late December, 1998, however, the first reports of local sleeping sickness transmission outside this area, in Soroti District, were recorded.

Soroti District lies to the north of the established T b rhodesiense focus in the south-east of the country. Although large tracts of the district were thought to be infested with the tsetse vector G f fuscipes, before December, 1998, only one case of sleeping sickness was recorded in the district in the mid-1960s.11 However, follow-up investigations concluded that this case most likely acquired the infection in the endemic area to the south. The first case in the present Soroti District outbreak was reported on Dec 31, 1998, and after identification of G f fuscipes in the area, tsetse control measures were implemented from February to July, 1999. Despite these measures, the outbreak persisted, and between Dec 31, 1998, and June 2, 2000, a total of 119 cases were recorded.
Understanding what triggered the Soroti District outbreak is important in both controlling the current outbreak, and preventing future outbreaks in other tsetse-infested but disease-free areas. Soroti experienced a period of major civil unrest at the end of the Ugandan civil war in 1979, leading to mass movement of people and livestock out of the district. More recently, since the return of civil stability, there has been a net migration of people back into the region and Soroti District has been the target of major rural development activities financially supported by both foreign donors and the Ugandan government. Central to these rural development activities have been large-scale cattle restocking programmes, undertaken without any surveillance of trypanosome prevalence in the cattle moved in the restocking exercise.

Our investigations of the origin of the Soroti District outbreak centred on the role of the domestic livestock reservoir. We investigated the hypothesis that the outbreak resulted from the importation of cattle infected with *T. brucei* rhodesiense into Soroti District from outbreak areas to the south, leading to local transmission around the Brookes Corner market. We aimed to identify the origin of cattle imported into Soroti District and traded at Brookes Corner market, and used a matched case-control study to assess the distance from Brookes Corner market as a risk factor for sleeping sickness, and detect spatial clustering of sleeping sickness cases over the initial period of the outbreak before any control activities.

**Methods**

**Determination of cattle origins**

District veterinary officials routinely recorded the district of origin, as well as the owner of each animal traded at Brookes Corner market. These written market records were cross-checked for the 4 years (April, 1995, up to and including December, 1998) preceding the current outbreak by consultation with traders and officials at Brookes Corner market.

**Case-control study**

We did a retrospective matched case-control study in June, 2000 to investigate whether the distance to Brookes Corner market was a risk factor for sleeping sickness.

All 119 sleeping sickness cases recorded between Dec 31, 1998, and June 2, 2000, came from within the catchment area of Serere Health Centre (figure 1). We recruited patients with sleeping sickness with complete records detailing age, sex, date of admission, and village of residence at time of contracting the disease. A total of 119 (mean 29·8 years, SD 19·9 years, 60% male) of 119 patients with sleeping sickness met these selection criteria.

Detailed patient histories of all 119 patients revealed that none had a history of travel outside Soroti District during the likely time at which the infection was acquired. Each case was matched with one control by age (<1, 1-9, 10-14, 15-19, 20-49, 50-64 and ≥65 years), sex and month of admission to Serere Health Centre. Patients diagnosed with any vector-borne disease were excluded, given the possibility of similar vector habitat biasing the results. We also excluded patients as controls if a definitive diagnosis or details of village of residence were not reported or if they had been referred from another health unit. All controls resided within the Serere Health Centre catchment area, which is heterogeneous in terms of ethnicity, the principal occupation of the inhabitants (subsistence agriculture), livestock production systems, and other population characteristics.

**Geographical data**

We recorded village location for each participant with a hand-held Global Positioning System (GPS, Garmin, Olathe, KS, USA). All GPS readings were taken at the central meeting point of each village as indicated by the village chairman. The locations of Brookes Corner market and Serere Health Centre were also recorded. The error in the GPS readings was less than 20 m.

All case and control geographical locations were entered into the ArcView (version 3·1) Geographic Information System (ESRI Systems, Redlands, CA, USA), in which Euclidean (straight line) distances from each case and control to Brookes Corner market were calculated.

**Statistical analyses**

We did an analysis of covariance (ANCOVA) using GLIM (version 4) to determine how the distance of cases and controls to Brookes Corner market (km) varied with time since the start of the outbreak. Case-control status was defined as a categorical variable with two levels, while time (in days) since the start of the outbreak on Dec 31, 1998, was judged a continuous covariate. Significance of the model was tested using an F test and the residuals were inspected for normality.

We undertook conditional logistic regression with the PROC PHREG procedure in SAS (version 8) to test whether distance from Brookes Corner market was a risk factor for sleeping sickness, and whether this changed over time course of the outbreak. The case-control status was used as the response variable, a unique number (1-113) was used to identify each pair of matched cases and controls, and the distance to Brookes Corner market (km), the time (in days) since Dec 31, 1998, and the interaction between the two were fitted as continuous explanatory variables. We tested for significance by comparing the reduction in deviance against the value in the χ² table.

**Cluster detection**

We used the spatial scan statistic to determine the most significant spatial cluster of cases, if any, over the first 32 days of the outbreak, from the first recorded case just before vector control was implemented in February,
**Results**

Of the recorded 2976 heads of cattle traded from April, 1995, to December, 1998, at Brookes Corner market, 54% (1510) originated from within the *T b rhodesiense* endemic sleeping sickness focus in the south-east of Uganda. This is an average of 18 animals per weekly market day sourced from sleeping sickness endemic areas.

Figure 2 shows the results of the ANCOVA. The minimum adequate model (*F* ratio=131.3, *df*=22,223, *p*<0.001) contained significantly different intercepts (*F* ratio=95.1, *df*=1,224, *p*<0.001) for the cases (3-70 km, 95% CI 2.22-5.18 km) and controls (10-22 km, 8.44-12.00 km), and a positive slope for distance of cases from Brookes Corner market with respect to time since the start of the outbreak (0.014 km per day, 95% CI 0.008-0.020 km per day, *F* ratio=17.5, *df*=1,224, *p*<0.001). The slope for distance of controls from Brookes Corner against time was not significantly different from zero and was removed from the model without any significant loss of explanatory power (*F* value=0.45, *df*=1,224, *p*>0.05).

When tested by itself using conditional logistic regression, increasing distance from Brookes Corner market was highly significantly associated with lower risk (*χ²*=69.30, *df*=1, *p*<0.001). Distance from Brookes Corner market was therefore a significant risk factor for sleeping sickness. Time since start of the outbreak (in days), when tested individually, was not significant (*χ²*=0.168, *df*=1, *p*>0.05). When both continuous variables (distance and time) and their interaction were tested together, the statistical model was highly significant (*χ²*=81.98, *df*=3, *p*<0.001) and could not be simplified by removal of the interaction term without a significant loss of explanatory power (*χ²*=12.60, *df*=1, *p*<0.001). The table shows the parameter estimates for this model. These parameters suggest that there is no change in overall risk through time, but that the spatial distribution of risk does change with time, moving away from the market.

Figure 3 shows the statistically significant cluster (overall relative risk=1.93, log-likelihood ratio=30.19, *p*<0.001) detected by the spatial scan statistic. The cluster, with a predicted radius of 4.2 km, included Brookes Corner market and 27 of 28 of cases recorded over the first 32 days of the outbreak (before implementation of control activities).

**Discussion**

Preventing the spread of sleeping sickness is important in averting the considerable disease burden and the high control costs associated with sleeping sickness. The results of our investigations on the causes of the outbreak suggest an association between the introduction of human-infective parasites to the new disease focus and the movement of cattle into the area from regions where sleeping sickness is endemic.

The outbreak was initially centred close to Brookes Corner market. The clustering of cases detected in the first month, before tsetse fly control, is consistent with tsetse fly acquiring infections near the market and transmitting the disease in the local area. The market and the surrounding landscape lie within an area of tsetse fly habitat, and, after sale, most of the animals were taken to homesteads close by. Although there is another market within the catchment area of Serere Health Centre, it is over 20 km from Brookes Corner and there was no association between this market and sleeping sickness cases. Our analyses suggest that sleeping sickness spread outwards from the initial focus during the time of the study. How far that spread will continue remains to be determined.

The movement of infected people into Soroti is unlikely to have resulted in the introduction of the parasite, as none of the cases of sleeping sickness at Serere Health Centre had a history of travel outside of the district. In addition, the prevalence of *T b rhodesiense* in cattle is much higher than that in human beings, and tsetse blood meal analysis shows that *G f fascipes* is much more likely to feed on cattle than on human beings. Molecular characterisation of *T brucei* species isolates from Soroti,
and their comparison with isolates from the known endemic and non-endemic areas, allowing detailed studies on the population genotypes of the parasites involved in the outbreak, is in progress. Material for genetic studies is available from all the recorded human cases and from cattle living in the vicinity of Brookes Corner market throughout the outbreak. There was, however, no screening of cattle brought to the market itself at the start of the outbreak.

Given that the restocking activities in Uganda are being implemented on a country-wide scale as part of a long-term poverty eradication plan, it is conceivable that sleeping sickness could spread to other tsetse-infested districts of Uganda as a result of cattle movements. The region around Lake Kyoga where this outbreak occurred, and beyond, is well endowed with riverine forest vegetation ideal for G. f. fuscipes; much of this habitat became established during the times of civil unrest when the area was depopulated and left uncultivated, and reverted to a bush habitat more suitable for the fly. Since then, the return of the human population has led to significant contact with this habitat and a large increase in the cattle population from restocking. The resultant interactions between human beings, their livestock, and the tsetse-infested environment led to an increased risk of introduction for parasites being transmitted. The continued spread of sleeping sickness would be expected in an area such as this, where marginal lands are being exploited.

Vector control was undertaken at the very start of the outbreak, and although such activities remain an option for sleeping sickness control in the area, they might not be cost-effective to maintain in the resource-poor rural communities affected by sleeping sickness. In particular, the cost of extending vector control over a larger area may become prohibitive and unsustainable. We suggest that the treatment of cattle is an important option for preventing importation and transmission of human-infective parasites in new areas. Control strategies such as the obligatory testing and treatment of cattle with a single curative dose of a trypanocide before sale in markets need to be evaluated, and the cost-effectiveness of such interventions must consider both medical and veterinary inputs and outcomes. The implementation of integrated control, targeting tsetse, cattle, and human beings, would benefit from coordination between veterinary and medical authorities. Such intersectoral collaborations would lead to improved human and animal health, and contribute generally to rural development in tsetse-infested countries across East Africa.

Contributors

The study was developed by M Odiit, E M Fevre, P G Coleman, S C Welburn, and M E R J Wooldhouse, and carried out by E M Fevre, J W Maguare, P G Coleman, and M Odiit. P G Coleman, E M Fevre, and M Odiit carried out the data analyses, and all authors were involved in data interpretation and report writing.

Acknowledgments

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References

A treatment centre in Serere has been handling at least 60 cases annually

Sleeping sickness hits Soroti

By Charles Wendo

As traders and farmers herded cattle from Busoga into Teso to replace those stolen by Rastafarian warriors, they carried sleeping sickness along. This is what a team of British and Ugandan researchers have proved of the epidemic that is now affecting many in Soroti, who had never seen it before.

The researchers now say that unless action is taken, cattle can spread sleeping sickness across the country. Their evidence has been published in the Lancet, a prestigious medical journal based in London.

Known as sleeping sickness in human beings and nagana in cattle, this is one of the diseases shared between man and beast. In both species the alternative name for the disease is trypanosomiasis.

According to the researchers, infected cattle were transported to Soroti district. Tsetse flies then peddled the sleeping sickness germs, scientifically known as Trypanosoma brucei rhodesiense, from cattle to man.

The research team was headed by Eric Fewe from the University of Edinburgh, UK. The Ugandans in the team were Dr. M. Odili and Dr. J.W. Maguwa, both from the Toro-based Livestock Health Research Institute.

"Given that restocking activities in Uganda are being implemented on a country-wide scale as part of a long-term poverty eradication plan, it is conceivable that sleeping sickness could spread to other tsetse-infected districts of Uganda as a result of cattle movements," the researchers wrote.

They found out that more than half of the cattle sold in the areas where sleeping sickness had broken out in Soroti District, originated from Busoga.

Dr. Dawson Mbulamberi, assistant commissioner in charge of vector-borne diseases, confirmed that sleeping sickness which has been lingering in Busoga since the 1970s, spread to Soroti after the restocking exercise begun.

A treatment centre in Serere has been handling at least 60 cases annually for the last three years. But Mbulamberi says this is an under-estimation since many patients do not go to the health centre for treatment.

Mbulamberi said the Ministry of Health would work closely with the veterinary department to control sleeping sickness.

"We have recommended that all cattle sold in markets should be treated for trypanosomiasis. This charge can be put on the animal owner. We shall have to get a policy passed through Cabinet so that it becomes a law," he said.
Tackling trypanosomiasis

Environmental aspects of insecticide use
Programme against African Trypanosomiasis (PAAT)

Plus poster
Sleeping sickness: a tale of two diseases

Susan C. Welburn, Eric M. Févre, Paul G. Coleman, Martin Odit and Ian Maudlin

Sleeping sickness presents clinically as two distinct diseases, reflecting the fact that two very different trypanosomes are responsible. The African Rift separating East and West Africa defines the distribution of the two diseases. In this review, Susan Welburn, Eric Févre, Paul Coleman, Martin Odit and Ian Maudlin discuss the biology and distribution of these two diseases in relation to the evolution of hominids in Africa.

African trypanosomiasis has re-emerged as a major health threat to rural Africans, with an epidemic, extending from the southern part of Sudan through Uganda and Congo to Angola, resulting in >100 000 new infections per year. The disease takes two forms in humans, presenting with distinct clinical pictures. Gambian sleeping sickness has a long asymptomatic stage, which is eventually succeeded by a subacute febrile illness followed by late-stage chronic meningoencephalitis; death might occur several years after onset of the disease. Rhodesian sleeping sickness progresses much more rapidly, with >80% of deaths occurring within six months of the onset of illness. These clinical differences are reflected in the methods adopted to control the disease. The control of Gambian sleeping sickness relies on case identification (principally using the card agglutination test) and chemotherapy of the largely asymptomatic human reservoir. Rhodesian sleeping sickness is zoonotic, with a reservoir in wild animals and in domestic livestock, which, combined with the acuteness of the disease, demands a much more aggressive response, including control of the tsetse vector (Box 1). Previously, Rhodesian sleeping sickness epidemics have even led to the large-scale removal of human populations at risk; in recent times, large-scale tsetse control operations, including aerial spraying, have been deployed together with medical interventions. The present transcontinental epidemic is almost exclusively of the Gambian form of the disease, a serious epidemiological problem.
Box 1. The animal reservoir and strategies for sleeping sickness control

Differences in the epidemiology of Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense might have profound effects on the relative effectiveness of sleeping sickness interventions. We have used a mathematical model of sleeping sickness transmission to examine how the effectiveness of different interventions – early curative treatment of humans, chemoprophylactic treatment of animals and vector control – will vary between the two species under different assumptions of the existence of an animal reservoir.

Gambian and Rhodesian sleeping sickness do show different clinical manifestations, particularly in the duration of infection (and so infectiousness). In the absence of any treatment, the natural duration of infection of Rhodesian sleeping sickness is, on average, about six months\(^8\), which is much shorter than the average two-year duration of untreated Gambian infections\(^9\). In the model used here, the basic reproduction number of infection, \(R_0\) (that is, the number of secondary infections arising from one infectious host in a population of fully susceptible hosts), is linearly related to the duration of infectiousness, such that \(R_0\) increases with increasing length of infectiousness.

Although the role of a non-human animal reservoir in \(T. b. rhodesiense\) transmission has long been recognized\(^2\), the importance of non-human hosts in the maintenance of \(T. b. gambiense\) is more uncertain. Rogers\(^3\) argued that domestic animals might be essential in the maintenance of \(T. b. gambiense\) because \(R_0\) in humans alone might fall below unity. This led to suggestions that the existence of a non-human animal reservoir in \(T. b. gambiense\) infections might have contributed to the failure of human population surveillance and treatment campaigns to eradicate sleeping sickness in certain settings\(^4\). However, it has also been argued that the existence of an animal population on which tsetse flies preferentially feed might result in humans receiving infectious bites at a lower rate than when flies feed only on humans\(^5\).

Here, we have extended the multi-host model of African trypanosomiasis developed by Rogers\(^3\) to incorporate the effects of different control options (Fig. 1). Our outcome of effectiveness is the reduction in the effective reproductive number (i.e. the number of secondary infections resulting from a primary case in a population in which some individuals might not be susceptible) relative to no intervention (i.e. \(R_0\)). The results show that early case treatment is most effective against Gambian infections when there is no animal reservoir (Fig. 1a). The presence of an animal reservoir reduces the relative effectiveness more for Rhodesian than for Gambian infections because the duration of infectiousness is greater in the latter and so transmission in human accounts for a greater proportion of the total \(R_0\). In Fig. 1b, we see that chemoprophylaxis of animals is most effective for the Rhodesian form, which occurred in Uganda in the 1980s, is now largely under control.

The causative organisms, \(T. b. gambiense\) and \(T. b. rhodesiense\), are morphologically indistinguishable, but a glance at a distribution map of the disease reveals a clear-cut boundary that roughly follows the great geological divide of Africa, the Rift Valley (Fig. 1). This divide is closely bound up with the epidemiology of the disease, which might have been an important factor in the evolution of hominids.

Genetic comparisons

There are three tsetse-borne species of the subgenus \(Trypanosoma\): \(T. b. gambiense\), human infective and found in foci to the west of the Rift Valley; \(T. b. rhodesiense\), also human infective and found in both wild and domestic animals to the east of the Rift; and \(T. b. brucei\), non-human infective and found in animals across sub-Saharan Africa. All three species are morphologically indistinguishable. Before the advent of DNA technology, human infectivity of animal isolates of \(T. brucei\)'s I was determined using either human 'volunteers' or parasite survival in human serum\(^6\). Many attempts have been made to identify the factors in human serum that might cause lysis of non-human infective \(T. brucei\) parasites (for review, see Ref. 8). Recently, a single gene has been identified in \(T. b. rhodesiense\) that renders human
 serum-sensitive *T. b. brucei* clones resistant to lysis in human serum. The fact that this gene is not expressed in *T. b. gambiense* highlights the fundamental difference between these parasites.

Early attempts to differentiate *T. b. brucei* stocks using isoenzymes revealed that *T. b. gambiense* was genetically homogeneous, but this technique could not consistently distinguish *T. b. rhodesiense* from *T. b. brucei* stocks. Restriction fragment length polymorphism (RFLP) analysis has since confirmed the monophyletic nature of *T. b. gambiense* but, more importantly, has allowed the separation of field isolates of *T. b. rhodesiense* from non-human infectious *T. b. brucei*. The apparent genetic homogeneity within natural populations of both species of human infective African trypanosomes has stimulated debate on the genetic structure of *T. b. brucei* populations. The origins of this controversy lie in the fact that, although *T. b. brucei* stocks are able to mate and undergo sexual recombination in the laboratory, evidence of genetic exchange among field populations has never been convincingly demonstrated. This debate as to whether mating takes place at all in natural populations is of interest outside the confines of population genetics because, if recombination is a normal part of the trypanosome life cycle, then such a panmictic (randomly mating) structure would inevitably lead to the spread of human infectivity throughout Africa via the animal reservoir of *T. b. brucei*. Both Cibulskis and
Tibayrenc questioned the panmictic structure of trypanosome populations, suggesting that they were essentially clonal, with sexual recombination rarely, if ever, occurring. Between these apparently polar positions lay the suggestion that population structures seen in the wild were the result of the clonal expansion of a few genotypes during epidemics, but that frequent sexual recombination was the norm. Frequent sexual recombination was predicated on the assumption that all stocks examined were obtained during epidemics, and on a misunderstanding of the nature of sleeping sickness epidemics. Although disease outbreaks might have been considered epidemic by medical authorities, in reality they might have involved an incidence not much greater than the endemic situation at the time. (A disease is said to be endemic when constantly present to a greater or lesser degree in people of a certain class or in people living in a particular location; an epidemic is a widespread outbreak of an infectious disease where many people are infected at the same time.)

RFLP analysis led to the clear conclusion that T. b. rhodesiense and T. b. brucei had very different population structures. Recently, a study of the same isolates, using the more informative minisatellite marker system, confirmed the clonal nature of T. b. rhodesiense but, again, suggested that T. b. brucei populations are probably sexual in nature and that this is obscured by the clonal expansion of a few genotypes during an epidemic. However, we have no way of knowing what constitutes a T. b. brucei epidemic, as the natural hosts of this parasite (game and domestic livestock) are not normally screened, except under the exceptional circumstances of a human outbreak. A comparison of cattle isolates from adjoining areas endemic and epidemic for T. b. rhodesiense (western Kenya and eastern Uganda, respectively) found little difference in population structure of T. b. brucei.

Moreover, studies of trypanosome populations in Uganda have shown that only two closely related genotypes were present in humans over long periods of time. There is no clear evidence that T. b. rhodesiense mates in the wild nor that it simply 'switches' to and from being T. b. brucei.

Origins of human infectivity

The origin of human infectivity of African trypanosomes remains an intriguing question for evolutionary biologists and historians. The idea of a monophyletic origin for human infectivity came from isoenzyme studies that suggested that subspecies of T. brucei s.l. are the product of radiation from an ancestral group of T. b. rhodesiense-type parasites that are now found in East Africa. (A monophyletic group contains an ancestor and all its descendants.) RFLP analysis readily separated Ugandan T. b. rhodesiense isolates from T. b. gambiae, but the same study showed that T. b. rhodesiense isolates from Zambia were more closely related to T. b. gambiae than to Ugandan isolates of T. b. rhodesiense. The phylogenetics of the subgenus Trypanozoon remains the subject of debate, but the most recent authoritative study based on 18S rRNA sequences groups T. b. rhodesiense with T. b. brucei within a tight but undifferentiated Trypanozoon clade, again emphasizing the genetic homogeneity of the subgenus Trypanozoon. However, RFLP and minisatellite studies all suggest that human infectivity has evolved independently in T. brucei in eastern Africa on at least two occasions, forming the Ugandan and Zambian foci of T. b. rhodesiense.

Geographically, Uganda forms a meeting point of the two diseases, with ancient but active foci of T. b. gambiae in the north west and T. b. rhodesiense in the south east. Some confusion exists about the nature of the parasite involved in the first recorded major epidemic in Busoga in the south east, around 1900. This confusion seemingly arose from the differing opinions of members of the Royal Society Sleeping Sickness Commission sent to investigate the causes of the epidemic at the time. Castellani was in no doubt that there were two different diseases present at that time in Uganda. The first was similar to T. gambiense described by Dutton in West Africa and identified as trypanosoma fever by Castellani; the second, called sleeping sickness, presented with acute symptoms and was named T. ugandense.
Castellani. Trypanosoma fever was observed only rarely, often in immigrant Sudanese soldiers, whereas sleeping sickness was at the root of the serious epidemic among the population of Busoga. Bruce later considered the possibility of there being two diseases but concluded that trypanosoma fever was simply the first stage of sleeping sickness\(^\text{29}\), despite the fact that he observed three Sudanese who had bloodstream infections for long periods yet were on duty and were not ill (i.e. they had chronic \(T. b. gambiense\) symptoms). With hindsight, we can see that Castellani might have been correct: the Sudanese had probably carried chronic infections with them to southern Uganda from the ancient Sudanese \(T. b. gambiense\) focus that Castellani identified as trypanosoma fever. The examination of existing clinical records from this time of the great epidemic shows that \(T. b. rhodesiense\)-like symptoms were clearly observable in patients from Busoga (E.M. Fèvre, unpublished). The native Busoga people were indeed suffering from a different acute disease, identified as sleeping sickness. However, \(T. b. rhodesiense\) was not recognized as the causative organism of this form of sleeping sickness in Busoga until the 1940s (Ref. 30) and was thought to have been brought to Uganda from Zambia where it had been identified\(^\text{11}\) in 1910. However, as we have seen, isolates of \(T. b. rhodesiense\) from Zambia bear little genotypic relation to Ugandan isolates\(^\text{14}\). Furthermore, \(T. b. rhodesiense\) isolates from Busoga have remained genetically unchanged for several decades\(^\text{19}\).

**East- and west-side stories**

The recent DNA studies support the view formed by Dutton and Todd\(^\text{66}\) a century ago that no district in which sleeping sickness has once existed is known to become entirely free from the disease: These ancient foci exist to this day, with the disease advancing and contracting, a process dependent on several ecological and sociological factors. Again, Uganda provides good examples of the timelessness of these foci, with the \(T. b. rhodesiense\) focus in the south east based around Busoga and the \(T. b. gambiense\) focus in the north west around West Nile. It is the very antiquity of these disease foci that brings us to the most intriguing question concerning the distribution of this disease. Figure 1 shows a clear demarcation between \(T. b. gambiense\) and \(T. b. rhodesiense\), roughly following the north-south divide of Africa formed by the Rift Valley. How could this have come about?

The answer to this might lie in the evolution of hominids, which is intimately connected with the Rift. Five million years ago, the climate of the earth began to change, going through dry periods that reduced the forest and increased the savanna areas in East Africa. The forest habitat of our human ancestors, the apes, would have been reduced, forcing those that lived in the eastern part of the continent to forage in open country. This change of habitat resulted in the evolution of bipedalism and eventually the evolution of hominids in the Rift Valley\(^\text{23-34}\). This change of habitat would also have brought the apes and early hominids into contact with a new set of parasites circulating in savannah-adapted game animals, in particular the trypanosomes. Savannah populations of \(T. brucei\) at some time in evolution would have acquired the gene(s) encoding human serum resistance and become a threat to the health of early hominids. \(T. b. rhodesiense\) is known to be a zoonotic parasite, having been transmitted experimentally from both wild\(^\text{6}\) and domestic\(^\text{6}\) animals to humans. We can assume that the apes had become adapted, over long periods, to \(T. b. gambiense\) in the forested areas to the west of the Rift Valley. Experimental work bears this out, and susceptibility of apes to \(T. b. gambiense\) and \(T. b. rhodesiense\) has been shown to vary between primate species. Several studies\(^\text{15-18}\) in which different species of primate were infected with trypanosomes show that the death rate for untreated animals infected with \(T. b. rhodesiense\) reaches 99%. Infections with \(T. b. gambiense\), by contrast, usually lead to chronic infections and long-term survival of the animal in question\(^\text{19}\). In experiments testing the serum sensitivity of a \(T. b. gambiense\) clone, serum from five ground-dwelling primates (two species of baboon, a mandrill, a gorilla and humans) gave similar results, indicating a period of common evolution and exposure to the parasite\(^\text{20}\). The significance of the animal reservoir for \(T. b. gambiense\) remains unclear: evidence of an animal reservoir for this trypanosome is based on isoenzyme analysis, \textit{in vitro} tests\(^\text{42}\) and RFLP techniques\(^\text{43}\) but, as far as we are aware, human experiments have never been attempted. \(T. brucei rhodesiense\) causes an acute disease in apes and monkeys as well as in humans and, we can assume, was also rapidly fatal to the evolving populations of early hominids. We know from DNA studies that human serum resistance evolved at least twice in \(T. brucei\), resulting in genetically different populations of \(T. b. rhodesiense\) in southern and eastern Africa\(^\text{18}\). Unlike other important parasites such as malaria, there is still no evidence for the evolution of any heritable immunity to \(T. b. rhodesiense\) in humans [although we are not aware of studies of major histocompatibility complex (MHC) distribution in humans in relation to sleeping sickness]. The only strategy early hominids could adopt in these circumstances would have been the avoidance of tsetse-infested areas. In so doing, they might have inexorably been forced out of the Rift Valley and so 'out of Africa'. We know that early human populations remained small for long periods of time in Africa and only started to increase rapidly in number on leaving the continent\(^\text{44}\). Humans settling in West Africa would have carried with them partial tolerance to \(T. b. gambiense\), which remains largely a chronic disease; it has been pointed out that humans...
and their closest relatives are essentially trypanotolerant to *T. b. gambiens*. In the east of sub-Saharan Africa, population pressures and agricultural practices would eventually have reduced or removed threatening tsetse populations sufficiently to allow long-term settlement there, despite the continued presence of an animal reservoir of *T. b. rhodesiens*.

However, recent evidence shows that the ranges covered by the two human infective subspecies might be approaching each other\(^1\), both types of disease are re-emerging and expanding in range. Given the clonal nature of trypanosome populations, it is unlikely that human infectivity would have spread across Africa as a byproduct of mating between infective and non-human infective members of the *Trypanosoma* subgenus. As we have seen in the past century, epidemics of sleeping sickness of either form arise at intervals over time as human and tsetse populations come into conflict\(^2\).

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muMT mice with parasite-specific immunoglobulin 1 purified from the serum of resistant mice prevented worm establishment, thus clearly indicating the role of antibodies in disease evasion. SHK

**Bednets and teachers controlling malaria**

While waiting for the development of an effective malaria vaccine, attention has focused on prevention and treatment of the disease. Intervention strategies to reduce malaria transmission rely upon the use of insecticide-treated bed nets (ITBN) and residual house spraying (RHS). In a comparative study in South Africa, the use of ITBN, although more costly, was significantly more effective in preventing malaria than RHS (Goodman, C.A. et al. 2001 Trop. Med. Int. Health 6, 280–295). Instead of relying upon government-instituted medical surveillance, an increase in public awareness appears to be essential, which could be further implemented through the health and educational authorities. From a recently published study carried out in Tanzania, it was apparent that school teachers could play an important role in diagnosing malaria. The presence of symptoms such as headaches, joint pains, muscle pains, feeling feverish and measuring oral temperatures in schoolchildren were used to diagnose malaria. Over 70% of children diagnosed by teachers were positive for malaria (Magnussen, P. 2001 Trop. Med. Int. Health 6, 273–279). Children diagnosed with malaria were subsequently treated during school time. The authors conclude that teachers could play a major and vital role in school health programmes. TS

**Ticks and climate change**

From the mid-1980s, the incidence of tick-borne encephalitis (TBE) around Stockholm substantially increased, as indicated by serological tests on all cases of encephalitis from 1980 to 1998. This rise is significantly related to a combination of milder winters and extended autumns (temperatures of 5–9°C), thus favouring spring development of the vector *Ixodes ricinus*. The highest mean temperatures recorded in the northern hemisphere occurred in 1990, 1995 and 1997. Since the mid-1990s, spring has arrived in Sweden about 12 days earlier than it did in the 1980s.Ticks become active when night temperatures rise to above 4–5°C. Blood-sucking larvae, nymphs and adults can become infected with TBE virus or carry other zoonoses. Although the presence of more people in endemic areas and an enlargement of host reservoirs (e.g. roe deer) might have influenced the figures, the expansion in the tick population might actually be underestimated as a TBE vaccine has been available since 1986 and the public is more aware of the disease. [Source: Reuters Health Online via ProMED-mail; E. Lindgren and R. Gustafson 2001] 

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**More thoughts on the control of trypanosomes in cattle**

In a recent letter to *Trends in Parasitology*, Coles¹ questioned the need to control trypanosomiasis in domestic cattle in sub-Saharan Africa, suggesting that game animals could be farmed instead. Game animals are naturally resistant to most economically important trypanosome species and, he argues, such resistance would remove the need for using treatments aimed at killing flies and/or parasites. As Coles points out, the elimination of the tsetse vector of trypanosomes on a large scale might not succeed for many biological, logistical and economic reasons.

Although the production of game animals is a reality in some parts of Africa (e.g. South Africa) and elsewhere (e.g. Canada), these activities usually take place on a large scale, intensively managed commercial farms. Although there might be financial gains to be made for some, such systems are entirely unsuitable for small-scale, subsistence agriculturalist and pastoralist societies, and displacing domestic cattle in favour of tamed wild game, even in only a few areas, is unrealistic. This is because the farming of game animals is unlikely to satisfy the variety of economic needs met by cattle and cattle-derived products, including meat and leather production, but especially milk production, dung production (for both building and as a fuel source), traction and simply as a type of financial investment. Perhaps more importantly, cattle are an integral part of the social fabric of many societies in sub-Saharan Africa and have been so for many thousands of years².

With regard to the threat of developing drug resistance, it is more appropriate to advocate the optimal, rational use of existing treatments than to change entire well-established production systems.

Providing simple tools that would improve diagnoses and prevent the inappropriate use of existing drugs, and ensuring that reputable drugs reach the market in the first place³, would be more compatible with the needs of African farmers. There are also public-health implications for continued parasite-control measures in livestock; in particular, the reduction in prevalence of human-infective trypanosomes in the cattle reservoir host⁴. Even if game-animal production were to be socially acceptable in African rural communities, the impacts on animal health and on the productivity of concentrating game in enclosed areas might be substantial. They would have little chance to roam naturally, and from a human-health perspective, there are many zoonotic diseases that might be introduced as a result of the proximity to human settlements⁵. Trypanosomiasis itself is a good example: although wild game such as bushbuck are trypanotolerant, they are an excellent reservoir host for trypanosomes, and...
increasing their densities in tsetse-infested areas close to human habitation might have a serious impact on sleeping sickness incidence.

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Sterile insect release and trypanosomiasis control: a plea for realism

The January 2001 issue of Trends in Parasitology provided an excellent series of papers on several aspects of African trypanosomiasis and contained a poster about the Programme Against African Trypanosomiasis (PAAT); a campaign has now been established called Pan African Trypanosomiasis Eradication Campaign (PATTEC), which will be formally launched in October 2001. Most of the aspirations of PAAT are laudable, albeit self-evident. However, a programme that advocates Glossina eradication using the sterile insect technique (SIT) seems distant from reality. It is disappointing to see that PAAT, which includes scientists and concerned international organizations, is prepared to endorse eradication as a goal, and has persuaded African heads of state that it is feasible and presumably affordable. Janice Taverman quotes Guy Freeland’s response to Peter Holmes’ remarks in the 7th PAAT newsletter where Freeland criticizes the approach only to be roundly rebuked by Brian Hursey who quoted the problems of sleeping sickness as a justification for SIT supported by the success of SIT in screwworm.

The position of the WHO Expert Committee is distinct from that of PAAT. In 1996, the WHO Expert Committee reported concluded that the SIT was not relevant in terms of control of African Human Trypanosomiasis. The WHO 1988 report states that “Since eradication of tsetse populations is not considered realistic, the objectives of vector control campaigns in an epidemic focus is to reduce rapidly and drastically the vector population to a level at which disease transmission is significantly reduced or interrupted.”

Impregnated traps and non-residual ultra-low volume (ULV) insecticide application allied to diagnosis and treatment, controlled the Uganda sleeping sickness epidemic in the 1980s (Ref. 4,5). Hursey’s comments that the PAAT objective should be effectively sacrosanct and protected from “the personal agenda of scientists and donors…will contribute very little to direct disease elimination.” Surely, communities affected by sleeping sickness should not wait two to three years for sterile males to be bred, a situation where Freeland’s argument applies. Large-scale trials of SIT have failed in Nigeria, Burkina Faso, Tanzania and Ghana through lack of sustainability and through re-invasion. Only Zanzibar can claim any success. Glossina austeni was eradicated at a cost of US$7 941 000 between 1994 and 1997. Msangi et al. remarked that, while the cost was US$7 941 000, only US$5 788 000 was spent. However, not included in these costs were the original capital costs of establishing the mass rearing facility at Tanga, the earlier suppression studies on Zanzibar and support costs at the International Atomic Energy Agency (IAEA) in Vienna.

To boost the colony, 3 861 242 pupae were shipped from Vienna, but no costs of this effort are available. A calculation of the projected costs of a continent-wide SIT release programme to eradicate Glossina on the basis of the Zanzibar (Unguja Island) experience would suggest that the following should be considered. An assumption of 22 Glossina species, a cost per 1600 km² eradicated of US$5 700 000 (not including costs referred to above) and an area of tsetse infestation of 8.5-9.0 million km² (Ref. 7,8). The calculation discounts Zanzibar because G. austeni also occurs on the Kenya and Tanzania mainland, and taking the 8.5 million km² as the area infested (8.5 × 10⁶ × 5.7 × 10⁶) divided by 16000 = US$5 67 600 million. However, in many areas, up to four different species of Glossina coexist; hence if SIT-based eradication is envisaged, this cost will escalate, depending on which of the 22 species are present. The capital costs of Glossina factories have also yet to be calculated. Given the speed of progress on Zanzibar an equivalent calculation reveals that the timescale of the operation would be roughly three years to control one species in 1600 km² therefore, 3 years × 22 species × (8.5 × 10⁶) (divided by 16000 km²) = 350 000 years to eradicate 22 species.

PAAT should be reminded that Portuguese workers eradicated Glossina from Principe in 1905 using sticky backpacks on plantation workers. Harris was spectacularly successful in catching G. pallidipes in Zululand in 1921 (Fig. 1) and Morris controlled sleeping sickness in Ghana during 1938-1944 by bush clearing and later insecticide (DDT) impregnated traps. Overall, sleeping sickness was controlled by a variety of approaches, and the success was sustained until health services collapsed through under-resourcing and civil unrest prevailed. The expectation that SIT will contribute uniquely to the eradication of Glossina against the background and reality of the health services in sub-Saharan Africa is unrealistic—given the current problems of HIV, TB and malaria, and the likely success of achievable public health objectives for the eradication of Guinea worm and elimination of onchocerciasis and lymphatic filariasis as public health problems. In all these diseases, the involvement of communities is essential.
Sleeping Sickness Rediscovered

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This meeting focused on human sleeping sickness, which, despite improvements in diagnosis and control, has returned with a vengeance across central Africa.

Control Activities

National Programmes, Simon Van Nieuwenhove (WHO, Geneva, Switzerland) outlined recent trends in the distribution of human sleeping sickness, and the current extent and scale of Trypanosoma brucei gambiense infection. The disease is often consigned as a problem of the past, but its resurgence was displayed through a series of brief reports of the situation in the worst affected countries [The Democratic Republic of Congo (RDC), Angola, Sudan and Uganda], where existing surveillance strategies are failing to capture the true extent of the current epidemic. For example, within the RDC, over 100,000 new cases have been detected in the past six years—a quarter of these in 1997 alone. These cases were detected through an active surveillance system covering only 9% of the 13 million people considered to be at risk. War, civil unrest and human population movements are exacerbating the problem. The lack of sufficient funds and the logistical difficulties of working with deteriorating infrastructures in their respective countries were identified by Théophile Josanono (Angola) and Constantin Mlaka Mla Blenge (RDC) as critical constraints to the effective implementation of large-scale surveillance and early treatment activities.

International and Bilateral Agencies and Programmes. The response of international agencies to the resurgence of human sleeping sickness was also presented by representatives from WHO, European Union and Programme Against African Trypanosomiasis (PAAT). A failure in the 'co-ordination' of national programmes with bilateral initiatives implemented through non-governmental organizations (NGOs) was considered, by Pierre Cattand (WHO, Geneva), to be detrimental to the efficiency of control operations. The new WHO Programme against Human Trypanosomiasis (established 1995) is seen as providing a cohesive and co-ordinated direction to public and private stakeholders, so optimizing the efficiency of disease control at the national level.

NGOs. The presentations of the various NGO activities perhaps highlighted the lack of centralized co-ordination. Although the field activities of NGOs such as Medicins Sans Frontieres (MSF) and Fond Medical Tropicale (FOMETRO) are invaluable in the control of human sleeping sickness, the epidemiological data generated from such work could be better used if it were made available through a centralized database. Regional initiatives [such as the Organisation de Coopération et de Coordination pour la Lutte Contre les Grandes Épidémies en Afrique de l'Ouest (OCCGE)], and bilateral initiatives (including those funded by the French and Belgian governments) also play a role.

Control Tools

Diagnosis. Little has changed in the past decade regarding the technologies for trypanosomiasis control in the field. Philippe Büscher (ITM, Antwerp, Belgium) discussed improvements to existing diagnostic tools, emphasizing that efforts had concentrated on improving existing technologies such as blood smear, quantitative buffy coat technique (QBC), the card agglutination test for trypanosomiasis (CATT), ELISA, microcentrifuge and in vitro inoculation kits. Veerle Lejon (ITM, Antwerp, Belgium) went on to discuss tools for stage determination in making a diagnosis. The two methods are the detection in the cerebro-spinal fluid (CSF) of: (1) trypanosome specific antibody; and (2) IgM. Laurent Penchenier [Organisation de Coopération et de Coordination pour la Lutte contre les Grandes Épidémies en Afrique de l'Ouest (OCCGE)] compared the CATT test with the latex agglutination test for T. gambiense, while Andrea García [Office de Recherches Scientifiques Techniques Outre-Mer (ORSTOM)] looked at the problem of aparasitaemic individuals (who remain seropositive) in longitudinal surveys: do these people contribute to focus persistence?

Vector control. The different methods of tsetse control in Southern Africa were summarised by Peter van den Bossche [Regional Tsetse and Trypanosomiasis Control Programme (RTTCP), Harare, Zimbabwe]. The objective must be to involve communities in small-scale projects with appropriate technology, where a social approach must accompany the technical and economic aspects of the programme. Joshua Okoth (Livestock Research Health Institute (LURI), Tororo, Uganda) discussed human ecology and its relation to tsetse ecology, with reference to southern Uganda, suggesting that changing agricultural practices might result in periodicticity in Glossina fuscipes fuscipes.

Treatment and follow-up. Simon Van Nieuwenhove (WHO, Geneva) stressed that, to be most effective, a treatment regime should cure the patient quickly and avoid disability. With no ideal drugs available, and with commercial development of new compounds unlikely, as the market is too small to justify heavy investment in new drugs by the private sector, we are left with the old, toxic drugs. Production of suramin, most used in T. b. rhodesiense treatment, was to be stopped by the manufacturer, but requests from WHO and others managed to maintain production for humanitarian reasons. Even when drugs are available at the community level, they are often unaffordable (e.g. Nifurtimox costs US$ 20 for 21 days treatment, and Eflornithine US$ 1070 for 14 days intravenous treatment). Donors are needed, as local communities cannot afford these drug costs. Christian Burn (Swiss Tropical Institute, Basel) presented a possible method of reducing the treatment time of late stage trypanosomiasis, by changing the administration schedule of melarsoprol, to reduce hospitalization costs; and Sylvie Bisser, Institut voor Tropicale Geneeskunde, Antwerp (ITG) presented the results of a clinical trial in the RDC that showed that melarsoprol could be combined with nifurtimox. Another clinical trial, in northern Uganda [Dominique Legros, Groupe Européen d'Expertise en Épidémiologie Pratique (EPICENTRE)] found Pentamidine to be less effective than expected against T. b. gambiense, which raised the question as to whether drug efficacy is focus specific. Finally, Ron Kaminsky (Swiss Tropical Institute, Basel) discussed the tolerance of T. b. rhodesiense to Eflornithine (DFMO).

Public Health. Public heath aspects of human sleeping sickness in terms of community health care provision, were discussed by Claude Laveissière (OCCEAC) who emphasized that local knowledge is an essential part of disease
control. Paul Coleman (University of Edinburgh, UK) and International Livestock Research Institute, Nairobi) had modelled the human public health impact of controlling the T. b. rhodesiense reservoir in cattle. He suggested that targeting chemotherapy in reservoir populations can reduce significantly the number of cases of human sleeping sickness. Joseph Ndundu (Kenya Trypanosomiasis Research Institute, KETRI, Kenya) highlighted the techniques that can be bought into play to prevent an outbreak of sleeping sickness caused by T. b. rhodesiense.

Research

Parasite and Vector Biology. The neuro-pathology of sleeping sickness was discussed by Krister Kristenson (Karolinska Institute, Sweden) in terms of interactions between trypanosome-released molecules and the host defence system, and the effect that these might have on clinical features of the disease. Moiz Bashir (Karolinska Institute, Sweden) outlined the bi-directional signalling processes believed to be responsible for immune and neuronal dysfunction in the host. A trypanosome lymphocyte-triggering factor has been identified that stimulates production of IFN-γ, which then stimulates parasite growth. Stefan Magez examined the association between TNF-α induction and disease severity in mice. TNF-α was shown to be crucial for control of parasitaemia in infected animals but did not influence survival. TNF-α also appeared to be directly trypanocidal.

Alain Buguet (CRSSA, France) showed, using human patients, that as sleeping sickness progresses in severity, major disruptions occur in circadian rhythms, and that release of cortisol, growth hormone and prolactin are all affected possibly due to the release of active compounds by the host and/or parasite. Victor Pentreath (Salfford University, UK) showed that the gut is profoundly affected showing altered permeability in a mouse model of experimental sleeping sickness. Jerry Sternberg (University of Aberdeen, UK) used a mouse model to show that NO is involved in suppression of splenic lymphocyte function and dystherteriosis, which leads to anaemia. Philippe Vincendeau (Université de Bordeaux, France) showed that macrophages from infected mice produce NO, which is required for parasite killing. Little is known about the mechanisms of parasite death. Sue Webbum (University of Edinburgh, UK) showed that, after stimulation with a lectin to induce parasite death, several novel genes are upregulated in dying parasites. Knowledge of the hitherto unknown pathways that lead to cell death of these parasites might lead us to novel mechanisms of parasite elimination.

Progress in the development of the first generation genetic linkage map for T. brucei was presented by Mike Turner (Glasgow University, UK). Genetic mapping is important in that: (1) it compliments physical mapping and thus underpins genomic sequencing; and (2) it indicates functional (as opposed to structural) genomic organization, which allows the analysis of the inheritance of traits of medical and biological interest (e.g. human infectivity, drug resistance). The methodological approach relies on a large number of polymorphic markers generated using amplified fragment length polymorphism (APFL) analysis. In the FI progeny resulting from a cross between clones of two stocks of T. brucei, 16 linkage groups containing 155 polymorphic markers were identified.

Perhaps the most exciting new work presented was by Luc Vanhamme [Insti-tuut voor Tropicche Geneeskunde (ITG), Brussels], who described a series of elegant experiments, spanning a decade, which have led to the identification of the SRA gene that appears to confer resistance to human serum lysis. This gene for human serum resistance can be transferred to a previously susceptible parasite conferring the human resistance phenotype.

Philippe Truc (ORSTOM, France) described how new methods for diagnosis for T. b. gambiens sleeping sickness have been applied. It appears that parasites present during an infection might not be clonal and that patients might be carrying more than one clone of T. b. gambiens at any given time.

Much interest has been shown in the composition of surface coats of trypanosomes, both bloodstream forms and procyclic forms. Mark Carrington (Uni-ersity of Cambridge, UK) gave an overview of the role of the VSG, the predominant surface protein on bloodstream form trypanosomes, as a protective molecule. Similarly, Isabel Roditi (Universität Bern, Switzerland) described the surface composition of procycls (insect form parasites) and showed that this parasite has two types of coat protein, one of which ceases to be expressed after para-site establishment. Jan Van den Abbeele (ITM, Antwerp, Belgium) revisited differ-enisation in tsetse, and looked at the processes involved during maturation to mammalian infective forms in the salivary glands.

Drugs and Vaccines

C.C. Wang (University of California, USA) suggested that advances in our understanding of the African trypanosomes in recent years provide greatly improved opportunities for discovering more efficacious and safer new anti-sleeping sickness drugs in the 21st century. Felix Kuzoe discussed DFM0 and its use for treatment of T. b. gambiens. The drug is costly, and the regime unpleasant, but DFM0 remains the only drug that can be used to treat melanospor-resistant strains, and a lower-cost oral formulation of the drug is needed. Jorge Atoguida (Instituto de Higiene e Medicina Tropical, Lisboa, Portugal) described the successful treatment of infected mice with topical applications of melanospor. Bernard Bouteille (IEN, France) described experimental cure of mice with Megazol. Louis Maes (Tibotec, Belgium) highlighted the pros and cons of screening for new anti-trypanosomal drugs. Reto Brun (STI, Switzerland) discussed novel compounds that have been tested for anti-trypanosomal activity. KETRI is looking at a Chinese compound. SII 1029, using green monkey populations as models, but relapse has occurred in all treated animals. Novartis (Switzerland) is looking at CGP 40215, but have so far reported problems with stage II disease treatment, as the drug does not seem to cross the blood-brain barrier. Aciel Haemers (Université Instelling Antwerpen (UIA), Belgium) took a different perspective, and, rather than screening drugs for activity, is attempting to synthesise a custom-designed compound with a specific biochemical target, and test it both in vivo and in vitro. Mike Barrett (University of Glasgow, UK) discussed drug uptake via nutrient transporters, where it should be possible to transpose the part of the active compounds that enable novel compounds to cross the parasite membrane. Curtis Powell (Applied Im-munology Research and Development Corporation, USA) discussed the potential for using components of the flagellar pocket of trypanosomes as the basis of development of a vaccine for trypanosomiasis. Edith Authie (Centre de Coopération Internationale en Recherche Agronomique pour le Dévelop-pement (CIRAD), France) described a series of elegant experiments that show that immunization against cysteine pro-tees in cattle might assist the host in establishing resistance to the pathology normally associated with trypanosome infection.
WHO/TDR/CTD Round Table

Where do we go from here? A special session was chaired by Alvaro Moncayo (TDR, WHO, Geneva). The need for integration of trypanosomiasis control with the rest of the public health care system was emphasized by Constantine Mlaka (RDC) and by Dawson Mbulumberi (Uganda). For this, cheap and reliable diagnostic tools, as well as a proper evaluation of the relative merits of the existing tests, were considered essential. The question of integrating trypanosomiasis control with other diseases for surveillance and treatment purposes, making full use of the public health care system, was raised by David Molyneux (Liverpool School of Tropical Medicine, Liverpool, UK); such integration would result in sustainability. The already important role of NGOs should be extended, and integrated to maximize limited resources. Until country representatives ask their governments to make trypanosomiasis control a priority, the international community will not take more interest.

Without good epidemiological data on the extent of the problem of sleeping sickness within a country, control measures cannot be effective. Claude Lavéissière took an entomological viewpoint, suggesting that actual transmission sites, the relative importance of domestic and wild animals as reservoirs of infection, and the best risk indicators for disease contraction should all be investigated further. Mickey Richer [International Medical Corp (IMC), Los Angeles, USA] commented on the animal reservoir, in that diagnostic techniques for field testing animal infections are needed to determine the importance of this reservoir. Christophe Parquet expressed concern at the lack of knowledge of risk factors for disease development in seropositive patients. Simon Van Meirvenne concluded by emphasizing that sustainability must be kept in mind in drug, diagnostic test and vector control research.

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New Molecular Targets for Filariasis Drug Discovery

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The challenge of this meeting was to identify new chemotherapeutic targets from the myriad of parasite enzymes, receptors, genome data and metabolic pathways that have recently been identified. Onchocerca volvulus, Wuchereria bancrofti and Brugia malayi afflict more than 100 million people, worldwide. Filarial drug discovery research at WHO has three specific goals: (1) discovery of new macrofilaricides; (2) discovery of anthelmintics that effect sterilization of adult female worms; and (3) identification of new macrofilaricides, to combat any emergence of ivermectin resistance. Clinically, new drugs must be safe, curative, effective in few doses, cheap and chemically stable (K. Awadzi, Honho Hospital, Ghana).

Second Generation Drugs

Two groups of anthelmintics in common use today act at ligand-gated ion channels in the nematode nervous system. Levamisole exerts its effects at the nicotinic acetylcholine receptor (nAChR), and avermectins act as irreversible agonists at glutamate-gated chloride channels (Glu-Cl). Nematodes possess a variety of receptors, receptor subunits and channels, and their expression can be developmentally regulated. Using model organisms, it may be possible to determine which genes encoding nAChR, Glu-Cl and their subunits are expressed at specific life cycle stages. Functional expression of such ligand-gated ion channels can serve as assays to screen chemically modified avermectins for desirable pharmacokinetic properties. In Caenorhabditis elegans, co-expression of the B-subunit of Glu-Cl with the α-subunit can produce ivermectin-potentiated glutamate-gated channels (A. Wolstenhome, University of Bath, UK). The genetics of ivermectin resistance in C. elegans and veterinary parasites can lead to identification of resistance mechanisms and responsible genes, which have homologues in human filarial parasites (W. Grant, Flinders University, Australia). Transgenic strains of C. elegans expressing filarial targets will help to identify crucial genes. Imidazoles exert their antiparasitic effects by targeting tubulin (C. Lübeke, Makere University, Uganda). Benzimidazole (BZ) binds filarial tubulin and binding studies of tubulin of Haemonchus contortus show that binding constants of several new fluorinated derivatives of BZ correlate with anthelmintic potency at recommended therapeutic doses. However, mutation in few amino acids is correlated with drug resistance to BZ and clinical development of one of the most promising new macrofilaricidal imidazole derivatives, UM10078, was terminated because of potential carcinogenicity in animals.

Novel Targets

Intracellular bacteria have been detected in most filarial worms. The phylogeny of these organisms, Wolbachia spp, has been shown to be congruent with the host phylogeny. Since co-divergence of hosts and symbionts can result in evolutionary co-adaptations, as in the case of arthropods and their endosymbionts, several research groups suggest that by eradicating filarial endobacteria, parasites may also die (C. Bandi and C. Genchi, University degli Studia, Italy; and A. Bianco, Liverpool School of Tropical Medicine, UK). Evidence supporting this view is the observation that tetracycline therapy reduces the number of Wolbachia in filarial oocytes and embryos while inhibiting filarial embryogenesis.